PN-ABT-168
91321

2.H-04

Final Progress Report: October 1984 through September 1985

Title: Development of a Defined Diet for In-vitro Membrane Mass Rearing of the Tsetse Fly

Prepared by: J.R. DeLoach
USDA-ARS-SPA-VTERL
P.O. Drawer GE
College Station, TX 77841
FTS: 527-1484

The third and final year of activity concerning the development of an artificial diet for blood feeding arthropods has ended with mixed results about success. The fact that it is not possible to obtain a renewal from AID places restrictions on the future of this research. Although a semi-defined artificial diet was successfully developed and tested, the greatest breakthroughs will come only with an understanding of the nutritional importance of lipids isolated with all hemoglobin preparations. To this end a process for isolating nondenatured hemoglobin free of membrane lipids has been developed. A manuscript for publication is in preparation.

The African counterpart Mr. Geoffrey Kapatsa has one year to complete his PhD at Texas A&M University. Since funding is a problem, Mr. Kapatsa may not complete his degree by the time his funds expire in December 1986.

Attached you will find publications from this research which give greater detail of our 3 years of progress. The most significant findings are:

1. Hemoglobin and albumin components of blood provide nutritional adequacy for tsetse flies.
2. Tsetse and stable flies have glycosidases in the digestive tract for blood meal digestion.
3. Freeze-dried blood offers some alternatives to feeding fresh blood to flies.

cc: BOST
PPC/CDIE

1990

4. Additives and physical processes can improve the nutritional quality of freeze-dried blood.
5. Nutritional superiority of pig blood over cow blood may be due to the physical nature of phospholipids.
6. Nutritionally inferior cow blood may have high concentrations of C:18-3 fatty acid which interferes with the fly's physiology.

2. H - 04

Bull. ent. Res. **79**, 41–45
Published 1989

Comparison of lipid-free haemoglobin and stroma-contaminated haemoglobin diets for adults of *Stomoxys calcitrans* (L.) (Diptera: Muscidae)

G. KAPATSA*, G. E. SPATES, C. L. SHEFFIELD and J. R. DELOACH

US Department of Agriculture, Agricultural Research Service, Veterinary Toxicology and Entomology Research Laboratory, P. O. Drawer GE, College Station, Texas 77841, USA

Abstract

Adults of *Stomoxys calcitrans* (L.) that fed on stroma-contaminated haemoglobin plus bovine serum albumin had normal survival, fecundity and egg hatch. Feeding on haemoglobin from which the erythrocyte membrane stroma had been removed, together with bovine serum albumin, reduced fecundity to zero. The addition of membrane ghost preparation to a lipid-free haemoglobin and bovine serum albumin diet restored the essential nutrients for normal fly fecundity. For normal reproduction therefore, *S. calcitrans* adults require erythrocyte stroma.

Introduction

Artificial diets for blood-feeding arthropods have been reported. Diets for adults of the stable fly, *Stomoxys calcitrans* (L.), and tsetse fly, *Glossina palpalis palpalis* (Robineau-Desvoidy) were independently reported by DeLoach & Spates (1984) and Kabayo *et al.* (1985). Both laboratories reported the use of a bovine haemoglobin and bovine serum albumin diet which essentially replaced whole blood. Haemoglobin which was isolated in this laboratory and suspected to contain membrane stroma as a contaminant was used as one component of the diet. A commercially available bovine haemoglobin was also found to be suitable in dietary tests (Kabayo *et al.*, 1985). However, some commercial preparations of bovine haemoglobin were found to be totally unsuitable for either *S. calcitrans* or *G. p. palpalis* (DeLoach & Spates, 1984).

Although a semidefined artificial blood diet for stable flies and tsetse flies is known, a true holidic diet cannot be defined until the question of purity of the two basic components of the diets is known. It is highly probable that the haemoglobin used in the so-called College Station diet (DeLoach & Spates, 1984) and the haemoglobin used in the KT-80 diet (Kabayo *et al.*, 1985) are indeed contaminated with membrane erythrocyte stroma components. Moreover, it is unclear as to whether haemoglobin is required or whether the stroma contaminants are required by the flies. Since our laboratory has reported the development of a process to isolate ultrapure lipid-free haemoglobin (DeLoach *et al.*, 1986), it became possible to test the hypothesis of the importance of stroma in the diet of adult stable flies (Sheffield *et al.*, 1987; Sheffield *et al.*, in press).

* Deceased.

Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the US Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

Rec'd in SCI APR 10 1990

cc BUSTIP
CDIF

Feeding tests were conducted with adults of *S. calcitrans*, and the fecundity, percentage egg hatch and percentage survival of the flies were determined. Flies were fed diets consisting of lipid-free bovine haemoglobin, stroma-contaminated bovine haemoglobin and various stroma additives in combination with bovine serum albumin.

Materials and methods

S. calcitrans was obtained from a stock colony maintained at this laboratory since 1969 (Ivie & Wright, 1978). The larval rearing method was as described by Bridges & Spates (1983). Newly emerged flies were sexed and placed in cages (12 females and 6 males). Twenty-four hours post-emergence, flies were fed to satiation from dental rolls saturated with a particular diet and were then fed daily for 13 days. Egg collection was begun on the sixth day and continued to the 13th day.

Haemoglobin isolation

Bovine blood (500 ml) was collected in a heparinized container from Brahman × Hereford cattle. Erythrocytes were isolated by centrifugation (at 1000 *g*) and washed, three times each, in 5 vol. of 0·9% NaCl. The washed erythrocytes were divided into equal portions for further isolation of bovine haemoglobin.

Stroma-contaminated bovine haemoglobin was prepared from erythrocytes which were haemolysed by hypotonic dilution in distilled water. One vol. of washed erythrocytes was mixed with 3 vol. of water, and the haemolysate was centrifuged at 10 000 *g* for 20 min. The supernatant containing the haemoglobin contaminated with erythrocyte stroma was freeze-dried for storage before use in feeding tests.

Lipid-free haemoglobin was prepared essentially as described by DeLoach *et al.* (1986). Basically, erythrocytes were dialysed against hypotonic phosphate buffer until an osmolarity of 160–180 mOsm/kg was reached. Then, dialysed porous erythrocytes were ultrafiltered with a 0·1-μm-pore hollow-fibre unit. The ultrafiltrate containing stroma-free haemoglobin was then freeze-dried and stored at ambient temperatures.

Freeze-dried bovine blood and bovine plasma

Fresh bovine blood was collected with heparin in 500-ml batches. Blood was immediately shell-frozen and freeze-dried in a laboratory scale freeze-dryer. Dried blood was stored at ambient temperature until use. The freeze-dried bovine blood was stored at ambient temperature until it was reconstituted in distilled water for feeding tests.

Bovine whole blood was collected in heparin and centrifuged at 1000 *g* for 20 min. The plasma supernatant was saved and frozen for feeding tests.

Erythrocyte ghost isolation

Since the haemoglobin purification procedure described by DeLoach *et al.* (1986) yields two components, lipid-free haemoglobin and a membrane-rich fraction, the membrane fraction was further processed to yield essentially haemoglobin-free membrane ghosts. The erythrocyte ghosts from the haemoglobin ultrafiltration process was mixed with an equal volume of KH_2PO_4 (0·05 mM, pH 7·2) (adjusted to 160 mOsm/kg with NaCl) and centrifuged at 10 000 *g* for 20 min. The pellet was washed three additional times with 4 vol. of buffer. The resulting white ghost pellet was resuspended in 0·9% NaCl to yield a ghost concentration of 1×10^{11}/ml. Erythrocyte ghosts were enumerated in a particle counter equipped with a channel analyser (Coulter Electronics, Hialeah, Florida).

Feeding tests

Replicate groups (3) of adult flies (12 females and 6 males) were fed the following diets: (1) freeze-dried bovine whole blood reconstituted in distilled water, (2) stroma-contaminated haemoglobin (14 g%) plus bovine serum albumin (6 g%) in distilled water, (3) stroma-contaminated haemoglobin (14 g%) plus fatty acid free bovine serum albumin (6 g%) (Cohn Fraction V, Sigma Chem. Co., St. Louis, Missouri), (4) stroma-free haemoglobin (14 g%) plus bovine serum albumin, and (5) stroma-free haemoglobin (14 g%) plus fatty acid free

bovine serum albumin (6 g%). Fecundity, percentage egg hatch and percentage survival were calculated. Data were analysed by analysis of variance.

Feeding tests with erythrocyte ghosts preparations

Replicate groups (3) of adult flies (12 females and 6 males) were fed the following diets: (1) freeze-dried bovine blood reconstituted in distilled water, (2) stroma-contaminated haemoglobin (14%) plus bovine serum albumin (6%) in distilled water, (3) stroma-free haemoglobin (14%) plus bovine serum albumin (6%) in distilled water, (4) 1 vol. diet no. 3 plus 1 vol. erythrocyte ghosts, (5) 1 vol. diet no. 3 plus 5 vol. erythrocyte ghosts, (6) bovine plasma, (7) 1 vol. bovine plasma plus 5 vol. erythrocyte ghosts. Fecundity, egg hatch and survival were recorded. Data were analysed by analysis of variance.

Results

Flies fed on the control diet (freeze-dried blood) showed excellent fecundity (Table I). The stroma-contaminated haemoglobin plus either bovine serum albumin or fatty acid free bovine serum albumin were not significantly different with respect to fecundity, percentage egg hatch and percentage survival. The mean fecundity of 48·6 eggs/female per day attained with the former diet is 20% higher than that previously reported for the so called College Station diet (DeLoach & Spates, 1984).

The most important aspect of these feeding tests is the finding that flies fed a diet consisting of stroma-free haemoglobin in combination with bovine serum albumin had zero fecundity. Survival on this diet was also significantly reduced (Table I). These are the first

TABLE I. *Fecundity, egg hatch and survival of* Stomoxys calcitrans *adults fed on lipid-free haemoglobin*

Diet	No. of eggs/female/day	Hatch (%)	Survival (%)
Freeze-dried bovine blood	69·8*a*	97·0*a*	95·4*a*
Lipid-containing haemoglobin			
plus bovine serum albumin (BSA)	48·6*b*	86·6*b*	94·6*a*
plus fatty acid free BSA	47·2*b*	75·1*b*	93·0*a*
Lipid-free haemoglobin			
plus BSA	0*c*	0*c*	63·2*b*
plus fatty acid free BSA	0*c*	0*c*	59·7*b*

The figures are means (n = 21).
Figures in same column followed by a common letter are not significantly different (P<0·05).
BSA = Bovine serum albumin.

results describing the nutritional requirement for erythrocyte stroma in the blood diet of adults of *S. calcitrans*.

When lipid-free haemoglobin plus bovine serum albumin was combined with increasing amounts of purified erythrocyte membrane preparation, fecundity increased proportionally (Table II). With 5 vol. of membrane preparation, the fecundity was significantly higher than with 1 vol. of membrane. Survival increased significantly as the amount of membrane

TABLE II. *Fecundity, egg hatch and survival of* Stomoxys calcitrans *adults fed on membrane stroma and protein*

Diet	No. of eggs/female/day	Hatch (%)	Survival (%)
Freeze-dried bovine blood	72·6*a*	95·3*a*	99·7*a*
Lipid-contaminated haemoglobin + BSA	44·7*b*	93·1*a*	95·5*a*
Lipid-free haemoglobin + BSA	2·7*c*	0*b*	86·4*b*
(1 vol.) membrane	12·9*d*	1·4*c*	91·7*b*
(5 vol.) membrane	44·5*b*	45·8*b*	99·6*a*
Bovine plasma	27·5*e*	90·1*a*	81·2*b*
Plasma + (5 vol.) membrane	33·0*e*	94·1*a*	84·7*b*

The figures are means (n = 21).
Figures in same column followed by a common letter are not significantly different (P<0·05).
BSA = Bovine serum albumin.

suspension in the diet increased (Table II). Lipid-free haemoglobin plus bovine serum albumin plus 5 vol. membrane was not significantly different from stroma-contaminated haemoglobin plus bovine serum albumin.

When membrane suspensions were added to bovine plasma, no significant improvement in fecundity, percentage egg hatch or percentage survival were observed (Table II). In all feeding tests with lipid-free haemoglobin plus bovine serum albumin, egg production was 0–3 eggs/female per day, with very little variation. Lipid-free haemoglobin plus bovine serum albumin plus membrane suspension fed to flies resulted in a percentage hatch not significantly different from that of control flies fed on reconstituted freeze-dried whole blood.

Discussion

We report here for the first time that erythrocyte stroma is required by adults of *S. calcitrans* for normal fecundity, egg hatch and survival. These results demonstrate the essential nature of the erythrocyte membrane components on the flies. Previous methods to isolate lipid-free haemoglobin have resulted in either denatured haemoglobin (Riggs, 1981) or stroma-contaminated haemoglobin (Rabiner *et al.*, 1967). Thus, the isolation methodology for large quantities of lipid-free haemoglobin (DeLoach *et al.*, 1986) has proved useful.

Haemoglobin purity and its stroma content have been previously reported (DeLoach *et al.*, 1986). All the lipid-free haemoglobin used in these tests was from a lot known to be lipid-free. The stroma-contaminated haemoglobin has been previously shown to contain cholesterol, sphingomyelin, phosphatidyl serine and phosphatidyl ethanolamine (DeLoach *et al.*, 1986; Sheffied *et al.*, 1987).

The addition of membrane suspensions to lipid-free haemoglobin plus bovine serum albumin diets gave a significant improvement in fecundity (Table II). Survival of the adults also improved significantly. Contrary to these results, the addition of membrane suspensions to bovine plasma did not cause an increase in the fecundity (Table II). Thus, haemoglobin is required by the flies. Apparently, plasma alone provides sufficient nutrient so that fecundity is about three-quarters of that found with stroma-contaminated haemoglobin plus bovine serum albumin. Plasma alone has been previously shown to provide enough of the essential nutrients for *Glossina* to reproduce.

Protein and stroma are the major required nutrients in the diets of blood-feeding arthropods. However, other nutritional factors are known to play a role in meeting dietary needs. For example, a dialysable factor from haemolysed blood cells is required by *G. p. palpalis* (Kabayo & Taher, 1986). Flies that were fed diets which had been previously dialysed failed to reproduce and their reproductive systems showed signs of atrophy. Normal reproduction was achieved if the dialysable component was returned to the diet.

The essential dietary components for adults of *S. calcitrans* and possibly other blood-feeding arthropods comes from two classes of molecules in the blood, protein and stroma. The protein component consists of bovine serum albumin and haemoglobin (DeLoach & Spates, 1984; Kabayo *et al.*, 1985). Evidence presented here shows the importance of membrane components in the diet of *S. calcitrans*. Before a true holidic diet can be developed, the essential components in the erythrocyte membrane must be determined.

Acknowledgements

This research was partially funded by the Agency for International Development and conducted under Research Agreement #3108 with the International Atomic Energy Agency, Vienna, Austria. The authors thank Dr J. Olson for his graduate student advice to G. Kapatsa. This research is a memorial to G. Kapatsa.

References

BRIDGES, A. C. & SPATES, G. E. (1983). Larval medium for the stable fly, *Stomoxys calcitrans* (L.).—*SWest Ent.* **8**, 6–10.

DeLoach, J. R., Sheffield, C. L. & Spates, G. E. (1986). A continuous-flow high-yield process for preparation of lipid-free hemoglobin.—*Analyt. Biochem.* **157**, 191–198.

DeLoach, J. R. & Spates, G. E. (1984). Hemoglobin and albumin diet for adult *Stomoxys calcitrans* and *Glossina palpalis palpalis*.—*SWest. Ent.* **9**, 28–34.

Ivie, G. W. & Wright, J. E. (1978). Fate of diflubenzuron in the stable fly and house fly.—*J. agric. Fd Chem.* **26**, 90–94.

Kabayo, J. P. & Taher, M. (1986). Effect of dialysing diet components on the reproductive physiology of *G. p. palpalis*.—*J. Insect Physiol.* **32**, 543–548.

Kabayo, J. P., Taher, M. & Van der Vloedt, A. M. (1985). Development of a synthetic diet for *Glossina* (Diptera: Glossinidae).—*Bull. ent. Res.* **75**, 635–640.

Rabiner, S. F., Helbert, R. J., Lopas, H. & Freidman, L. H. (1967). Evaluation of a stroma-free hemoglobin solution for use as a plasma expander.—*J. exp. Med.* **126**, 1127–1142.

Riggs, A. (1981). Preparation of blood hemoglobins of vertebrates.—pp. 5–29 *in* Antonini, E., Rossi-Bernardi, L. & Chiancone, E. (*Eds*). Methods in enzymology. Volume 76. Hemoglobins.—874 pp. Orlando, Florida, Academic Press.

Sheffield, C. L., Spates, G. E. & DeLoach, J. R. (in press). Hypoosmotic dialysis and ultrafiltration tecnnique for preparation of mammalian hemoglobin: a comparison of three species.—*Biomater. Artif. Cells Artif. Organs.*

Sheffield, C. L., Spates, G. E., Droleskey, R. E., Green, R. & DeLoach, J. R. (1987). Preparation of lipid-free human hemoglobin by dialysis and ultrafiltration.—*Biotechnol. Appl. Biochem.* **9**, 230–238.

(*Received 3 May 1988*)

© C·A·B International, 1989

J. Insect Physiol. Vol. 35, No. 3, pp. 205–208, 1989
Printed in Great Britain

0022-1910/89 $3.00 + 0.00
Pergamon Press plc

2H-04

THE NATURE OF ERYTHROCYTE STROMA LIPID COMPONENTS REQUIRED FOR NORMAL REPRODUCTION IN *STOMOXYS CALCITRANS* (DIPTERA: MUSCIDAE)

G. M. KAPATSA✝, G. E. SPATES,* C. L. SHEFFIELD,* J. P. KABAYO† and J. R. DELOACH*‡

*U.S. Department of Agriculture, Agricultural Research Service, Veterinary Toxicology and Entomology Research Laboratory, P.O. Drawer GE, College Station, TX 77841 U.S.A. and †Entomology Unit, Joint FAO/IAEA Programme Agency's Laboratories, A-2444 Seibersdorf, Austria

(*Received* 6 *June* 1988; *revised* 17 *August* 1988)

Abstract—Adult *Stomoxys calcitrans* require erythrocyte membrane components in their diets for normal reproduction. Nutritionally sub-optimal diets consisting of stroma-free, lipid-free haemoglobin and serum albumin were greatly improved by supplementation with either erythrocyte ghost preparations or lipid mixtures containing cholesterol, sphingomyelin and phosphatidyl ethanolamine. The apparent nutritional requirement of dietary erythrocyte membrane components by adult *Stomoxys calcitrans* is discussed in terms of the nutritional value of the erythrocyte membrane lipid components.

Key Word Index: Lipids, arthropods artificial diet, nutrition, stable flies

INTRODUCTION

The development of semi-defined, nutritionally adequate, synthetic diets for blood-feeding insects (DeLoach and Spates, 1984; Kabayo *et al.*, 1985) has been a significant achievement in the quest for a full understanding of the nutritional requirements of these insects. The major components of these diets (haemoglobin and albumin) are heterogeneous macromolecules whose chemical composition and nutritional quality is dependent on a variety of impurities with which they are co-purified. The nutritional quality of serum albumin to tsetse flies has, for example, been associated with its bound lipid impurities rather than to the albumin protein *per se* (Kabayo, 1982).

Analysis of a haemoglobin preparation used in synthetic diet mixtures revealed a variety of lipid contaminants which are thought to originate from erythrocyte stroma components (Sheffield *et al.*, 1987). Recently, DeLoach *et al.* (1986) employed a dialysis ultrafiltration system to isolate large quantities of stroma-free, lipid-free haemoglobin; this made it possible, for the first time, to investigate the nutritional value of lipids normally bound to haemoglobin constituents of synthetic diets.

The present study was undertaken to examine the nutritional importance of erythrocyte stroma components in diets for *Stomoxys calcitrans*.

MATERIALS AND METHODS

Flies

S. calcitrans flies were obtained from a colony routinely maintained as previously described (Ivie and Wright, 1978; Bridges *et al.*, 1984). For each diet tested, three cages of newly emerged flies (6 males and 12 females/cage) were used.

Diets

Bovine haemoglobin was prepared as described by DeLoach and Spates (1984); bovine lipid-free haemoglobin was prepared using the dialysis and ultrafiltration methods described by DeLoach *et al.* (1986). Bovine serum albumin, fraction V, sphingomyelin from bovine brain, phosphatidyl ethanolamine, phosphatidylserine, cholesterol and choline chloride were obtained from Sigma Chemical Company, St Louis, MO. Sphingomyelin was 99% pure and contained primarily lignoceric acid, phosphatidyl ethanolamine and phosphatidyl serine were 98% pure. Cholesterol was 99% pure. All phospholipids as supplied were originally isolated from bovine tissue. Bovine erythrocyte ghosts were prepared from the pink erythrocytes remaining after the extraction of haemoglobin by the dialysis and ultrafiltration method (Sheffield *et al.*, 1987). The pink erythrocytes, containing less than 2% of their original haemoglobin, were washed 3 times, with 4 vol of hypo-osmotic phosphate-buffered saline (15 mM KH_2PO_4, 70 mM NaCl, pH 7.2, osmotic pressure 170 mOsm/kg), and centrifuged (10,000 *g* for 30 min) between washes. The resulting pellet of white ghosts was resuspended in 19 vol of isotonic saline and ghost-cell density of the suspension was determined with a particle counter (Coulter Electronics, Hialeah, FL). Using this procedure, the ghost-cell count of the suspension was 6–7 × 10^8 cells/ml.

A series of synthetic diets were prepared comprising bovine serum albumin (6.5%) and total haemoglobin (12%) with different proportions of bovine lipid-free haemoglobin and bovine haemoglobin (as shown in Table 1).

✝Deceased
‡To whom correspondence should be addressed.

cc BUSTIA
CPIE

in SCI APR 10 1990

Table 1. Survival and reproduction in *Stomoxys calcitrans* fed synthetic diets containing different proportions of haemoglobin-bound lipids

Diet composition* %(w/v in distilled water) BHb	LFHb	BSA	Hb-bound lipid impurities† (%)	Survival (%)	Number eggs female day	Hatch (%)
12	0	6.5	100	97.4 ± 2.6a	54.5 ± 4.7a	87.0 ± 6.4a
10	2	6.5	83	96.2 ± 4.0a	73.5 ± 3.4b	94.8 ± 0.9a
7	5	6.5	58	91.4 ± 3.7a	71.4 ± 3.1b	87.6 ± 6.5a
5	7	6.5	42	98.2 ± 2.0a	71.4 ± 5.3b	83.9 ± 6.4a
2	10	6.5	17	91.2 ± 4.7a	43.2 ± 3.3c	33.5 ± 4.1b
0	12	6.5	0	88.9 ± 4.2a	0.9 ± 0.4d	2.1 ± 1.5c

Data are $\bar{X} \pm$ SE ($n = 21$). Data in same column followed by a common letter are not significantly different ($P < 0.05$).

*Abbreviations: BHb: bovine haemoglobin, LFHb: bovine lipid-free haemoglobin, BSA: bovine serum albumin.

†Based on proportion of lipid-free haemoglobin to lipid-contaminated haemoglobin.

Diets containing different volume equivalents of erythrocyte ghost cells (shown in Table 2) were prepared by resuspending pellets of ghost cells obtained when the respective volume of ghost-cell suspension was centrifuged (10,000 *g*, 30 min). The ghost-cell pellets from different volumes were resuspended in a lipid-free synthetic diet (12% bovine lipid-free haemoglobin, 6.5% bovine serum albumin in distilled water).

Diets in which the lipid-free synthetic mixture was supplemented with various amounts of different phospholipids and cholesterol were prepared as follows: For each diet, the amounts of phospholipid or cholesterol shown (Table 3) were dissolved in chloroform-methanol (2:1, v/v) and rotor evaporated to dryness in a round bottom flask. 40 ml 6.5% bovine serum albumin in distilled water was then added to the flask and the lipids were then suspended by sonication (probe type sonicator, 100 W for 5–7 min). Haemoglobin (12% w/v) was then added and the diet volume adjusted to 60 ml. The resulting suspensions were used as the test diets.

Feeding tests

After preparation, the diets were stored frozen in appropriate volume aliquots sufficient for 1 day's feeding on each diet regimen. Diets were thawed on the day they were to be used. The flies were fed daily on small cotton rolls saturated with the respective diet immediately before feeding. The survival of the females, the number of eggs deposited by each female, and percentage of eggs hatched (between day 6 and day 12 of female life) were recorded for each diet tested.

RESULTS

A synthetic diet comprising bovine lipid-free haemoglobin and bovine serum albumin (negative control diet) does not meet the nutritional requirements for normal reproduction in *S. calcitrans*. Survival is not affected. Substituting a lipid-contaminated haemoglobin (e.g. bovine haemoglobin) for bovine lipid-free haemoglobin constitutes a diet which supports normal egg production and hatching (Table 1). Diets comprising a mixture of bovine haemoglobin and bovine lipid-free haemoglobin in various ratios support egg production and hatching in a manner commensurate with the proportion of the bovine haemoglobin component in such diets. Supplementation of the negative control diet with preparations of ghost cells (Table 2) restores its nutritional values in a dose-dependent fashion. A selection of specific lipids known to be present in ghost-cell membranes were tested singly or in combination for their capacity to improve the nutritional quality of the negative control diet to which they were added (Table 3). Sphingomyelin alone supported egg production but not egg hatch. Phosphatidyl ethanolamine alone supported egg production but to a lesser degree. Choline as supplied by either choline chloride or sphingomyelin supported egg production. Only a combination of cholesterol, a choline source and phosphatidyl ethanolamine supported egg production and egg hatch. Addition of another phospholid, phosphatidyl serine, to the aforementioned combination significantly improved egg production but not egg hatch.

Table 2. Effect of added erythrocyte ghost-cell components on the nutritional value of a lipid-reduced synthetic diet to *Stomoxys calcitrans**

Volume of ghost-cell suspension (ml)	Calculated concentration (% w/v) lipid-contaminated Hb	Survival (%)	Number eggs female day	Egg hatch (%)
0	0	86.7a	0.9 ± 0.4a	0a
1	0.95	77.4a	5.2 ± 1.1b	0a
4	3.80	98.6b	37.6 ± 4.0c	12.2 ± 8.2b
10	9.50	95.2b	35.8 ± 2.3c	71.2 ± 6.0c
15	14.25	95.4b	54.6 ± 4.4d	86.4 ± 6.2d
20	19.00	98.5b	45.3 ± 3.5d	91.2 ± 6.0d

Data are $\bar{X} \pm$ SE ($n = 21$). Data in same column followed by a common letter are not significantly different ($P < 0.05$).

*Pellet resulting from centrifugation of indicated volume of ghost-cell suspension was resuspended in lipid-free synthetic diet (12% bovine lipid-free haemoglobin, 6.5% bovine serum albumin, w/v in distilled water).

Table 3. Effect of lipid additives on the nutritional value of a lipid-free synthetic diet to *Stomoxys calcitrans**

Feeding test	Lipid additives (mg/100 ml) lipid-free synthetic diet)†					Insect survival (%)	Number eggs/female/day	Eggs hatched (%)
	SM	PE	PS	CH	Choline chloride			
1	0	0	0	0	0	78.1 ± 9.2a	0.4 ± 0.2a	0a
2	40	0	0	0	0	89.2 ± 3.7a	25.3 ± 3.3b	1.6 ± 1.6a
3	0	7	0	0	0	76.3 ± 4.2a	10.1 ± 1.6c	0a
4	40	7	0	25	0	93.2 ± 6.0b	28.3 ± 3.8b	82.5 ± 2.5b
5	0	0	0	25	40	72.1 ± 3.8a	26.8 ± 4.2b	0.9 ± 0.9a
6	40	7	40	25	0	92.1 ± 4.1b	36.1 ± 5.8d	73.0 ± 3.0b

*Data are $\bar{X}$ ± SE of 6 feeding tests. Data in same column followed by a common letter are not significantly different ($P < 0.05$).

†Abbreviations: SM: sphingomyelin from bovine brain, PE: phosphatidyl ethanolamine, PS: phosphatidylserine; CH: cholesterol.

DISCUSSION

In the present study, we investigated the nutritional importance of ghost-cell membrane components as the source of lipid contaminants of the haemoglobin constituent in synthetic diets. While methods have been developed for the delipidation of albumin without denaturing it (e.g. Kabayo, 1982) no delipidation method prior to the preparation of lipid-free haemoglobin (DeLoach *et al.*, 1986; Sheffield *et al.*, 1987) could facilitate evaluation of the nutritional importance of lipid contaminants in haemoglobin through diet-deletion techniques. Addition of lipid-contaminated haemoglobin to bovine lipid-free haemoglobin while keeping the total protein concentration constant (Table 1) is apparently equivalent to the addition of ghost cell components to bovine lipid-free haemoglobin. This is confirmed by the results shown in Table 2 where addition of preparations of ghost-cell membranes caused improvements in the nutritional value of bovine lipid-free haemoglobin similar to those caused by addition of bovine haemoglobin (Table 1). If one red blood cell contains about 14.6×10^{-10} g haemoglobin and the ghost-cell suspensions used in the present study were typically 6.5×10^{7} cells/ml, it can be estimated that 1 ml of such ghost cell suspension (Table 2) would normally have been associated with 0.95 g ($14.6 \times 10^{-10} \times 6.5 \times 10^{7}$) haemoglobin. Thus, the results in Tables 1 and 2 can be compared.

The amounts of the specific lipid additives used in the tests (Table 3) were similar to the levels reported for these components when DeLoach *et al.* (1986) analyzed lipid-contaminated haemoglobin preparations such as bovine haemoglobin. Sphingomyelin, which slightly improved survival in the present study, is a minor class of choline-containing phospholipids which has not yet been found in any dipteran (Fast, 1970). Most recently, Spates *et al.* (1988) has shown that stable flies utilize 98% of the ingested sphingomyelin found in a blood meal. Choline has been shown to be an essential dietary component for many insects and is known to serve micrometabolic and catalytic functions; it is also a lipogenic factor which plays an important structural and functional role as a constituent of the phospholipids (choline phosphatides) of cell membranes, transport lipoproteins and the neurotransmitter acetylcholine (Bridges, 1972). Thus, choline supplied as either choline chloride or sphingomyelin is essential to the flies. Since bovine erythrocytes contain no phosphatidylcholine, and since a diet comprising haemoglobin (contaminated with ghost-cell membrane components) plus bovine serum albumin is nutritionally adequate, we can also deduce that choline or its derivatives may be obtained from some other dietary lipid, e.g. sphingomyelin, which was here shown to improve survival but had no effect on fecundity. Dietary phospholipids provide saturated and unsaturated fatty acids. The improvement of adult viability caused by addition of animal-derived phospholipids to larval diets of the mosquito, *Culex pipiens*, could also be achieved by substituting arachidonic acid for phospholipids (Dadd and Kleinjan, 1979). Whether the observed improvement in survival in the present study was effected in this manner is yet to be determined. However, sphingomyelin used here contains approx 80% esterified ligoceric acid and 20% esterified stearic acid. It is possible that the improved hatch found with test No. 4 versus test No. 5 is due to the presence of fatty acids.

It is well established that insects are unable to synthesize the sterol nucleous and require dietary sterol which they utilize as structural components of cells and for the synthesis of ecdysoid growth regulators (Thompson *et al.*, 1972). In our studies, cholesterol with choline alone increased fecundity considerably but did not affect egg hatch. However, in combination with sphingomyelin, phosphatidylethanolamine and phosphatidylserine both improved fecundity and egg hatch. Tsiropoulos (1980) showed that although female olive fruit flies that were deprived of cholesterol laid a normal number of eggs, hatch was considerably reduced, and discovered that cholesterol improved hatch only when fed in combination with vitamins, amino acids, and minerals. Based on recent studies (Spates *et al.*, 1988) in our laboratory which showed 28% of ingested cholesterol to be utilized in eggs, we believe cholesterol to be an essential dietary constituent.

The results of this study are significant in as far as the work represents a step in current efforts to dissect the synthetic diet and to discern the dietary requirements of *S. calcitrans* and other blood-feeding arthropods. The ultimate goal is to use the information and conclusions drawn from such work as a basis for the construction of chemically defined diets.

Acknowledgements—The authors thank C. Ayala and H. Kirch for technical help. Agency for International Development for funding Geoffrey M. Kapatsa's graduate program and the International Atomic Energy Agency, Vienna, Austria for their support of John P. Kabayo. This paper is a memorial to Geoffrey M. Kapatsa's scientific effort and is our tribute to him as a valued colleague.

REFERENCES

Bridges R. G. (1972) Choline metabolism in insects. *Adv. Insect. Physiol.* **9**, 51–110.

Bridges A. C., Summerlin J. W. and Spates G. E. (1984) A new and more economical base medium for rearing larvae of the stable fly, hornfly, and housefly. *Sthwest Ent.* **9**, 388–391.

Dadd R. H. and Kleinjan J. E. (1979) Essential fatty acids for the mosquito, *Culex pipiens*. Arachidonic acid. *J. Insect Physiol.* **25**, 495–502.

DeLoach J. R. and Spates G. E. (1984) Hemoglobin and albumin diet for adult *Stomoxys calcitrans* and *Glossina palpalis palpalis* (Diptera: Glossinidae). *Sthwest Ent.* **9**, 28–34.

DeLoach J. R., Sheffield C. L. and Spates G. E. (1986) A continuous-flow high yield process for preparation of lipid-free hemoglobin. *Analyt. Biochem.* **157**, 191–198.

Fast R. G. (1970) Insect lipids. A review. *Prog. Chem. Fats Lip.* **11**, 181–242.

Ivie G. W. and Wright J. E. (1978) A fate of diflubenzuron in the stable fly and house fly. *J. Agric. fd Chem.* **26**, 90–94.

Kabayo J. P. (1982) The nature of the nutritional importance of serum albumin to *Glossina morsitans*. *J. Insect Physiol.* **28**, 917–923.

Kabayo J. P., Taher M. and Van der Vloedt A. M. V. (1985) Development of a synthetic diet for *Glossina* (Diptera: Glossinidae). *Bull. ent. Res.* **75**, 635–640.

Sheffield C. L., Spates G. E., Droleskey R. E., Green R. and DeLoach J. R. (1987) Preparation of lipid-free hemoglobin by dialysis and ultrafiltration. *Biotech. appl. Biochem.* **9**, 230–238.

Spates G. E., DeLoach J. R. and Chen A. C. (1988) Ingestion, utilization and excretion of blood meal sterols by the stable fly, *Stomoxys calcitrans*. *J. Insect Physiol.* **34**, 1055–1061.

Thompson M. J., Svoboda J. A., Kaplanis J. N. and Robbins W. E. (1972) Metabolic pathways of steroids in insects. *Proc. R. Soc. Lond. B* **180**, 203–221.

Tsiropoulos G. (1980) Major nutritional requirements of adult *Dacus oleae*. *A. Ent. Soc. Am.* **73**, 251–253.

BIOTECHNOLOGY AND APPLIED BIOCHEMISTRY 9, 230–238 (1987)

2. H- 04

Preparation of Lipid-Free Human Hemoglobin by Dialysis and Ultrafiltration[1]

CYNTHIA L. SHEFFIELD, GEORGE E. SPATES, ROBERT E. DROLESKEY, RALPH GREEN,* AND JOHN R. DELOACH

*Veterinary Toxicology and Entomology Research Laboratory Agricultural Research Service, United States Department of Agriculture, College Station, Texas, 77841, and *Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio, 44106*

SHEFFIELD, C. L., SPATES, G. E., DROLESKEY, R. E., GREEN, R., AND DELOACH, J. R. Preparation of Lipid-Free Human Hemoglobin by Dialysis and Ultrafiltration. *Biotechnol. Appl. Biochem.* 9, 230–238 (1987).

Dialysis of human red blood cells using a hypotonic solution and a commercial kidney dialysis unit followed by ultrafiltration through 0.1 μm pore hollow fibers provides an easily managed method for isolation of lipid-free hemoglobin. High pressure liquid chromatography analysis of lipid-free hemoglobin (LFHB) indicates 99–100% protein purity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis demonstrated that LFHB migrates as a single band. The process requires hypoosmotic dialysis of human RBC to a final 119–139 (av 132) mosmol/kg osmotic pressure. Additional reduction in osmotic pressure results in irreversible cell lysis which results in lipid contamination of the hemoglobin. Processing one-half liter of packed red blood cells requires 10 h, resulting in an average of 90% hemoglobin recovery. © 1987 Academic Press, Inc.

Hemoglobin is the major protein constituent of red blood cells and accounts for over 95% of the total soluble protein of the cell. Isolation of hemoglobin from red blood cells involves a simple procedure of hemolyzing the red blood cells followed by centrifugation to remove membrane stroma. Hemolysis results in a hemoglobin solution that is more than 90% pure (1). In some cases, organic solvents such as toluene have been used as hemolytic agents (2). However, such treatment can denature the hemoglobin (1).

Although stroma-free hemoglobin (SFHb)[2] has been evaluated as a blood substitute (3, 4), there exists no methodology to prepare large quantities of SFHb. Crystallization from a high concentration of phosphate solution requires several days and gives an average of 3% methemoglobin (5). Cheung *et al.* recently reported the preparation of SFHb by a DEAE-cellulose absorption technique (6). Rabiner *et al.* (7) used a 0.22 μm membrane to filter hemoglobin preparations and reported that preparations were stroma-free as evidenced solely on the lack of coagulant activity. However, lipid analysis was not conducted to determine the presence or absence of stroma.

[1] Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

[2] Abbreviations used: SFHb, stroma-free hemoglobin; DIC, disseminated intravascular coagulation; Hct, hematocrit; LFHb, lipid-free hemoglobin; PL, phospholipid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM, scanning electron microscopy.

0885-4513/87 $3.00

Copyright © 1987 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Rec'd in SCI APR 10 1990

Lack of coagulation activity was the only criterion used to determine the absence of lipids. This criterion is not conclusive because it has been shown that disseminated intravascular coagulation (DIC) attributed to stroma contaminants in hemoglobin preparations is not necessarily due to lipid contamination.

Rosenberry *et al.* (8) have approached the isolation of the other products from red blood cells, i.e., erythrocyte ghosts, by a high volume molecular filtration technique. Their process is dependent upon passage through a series of submicron filters (100,000 Da). The process requires 2 h and is limited to very dilute suspensions of red blood cells [hematocrit (Hct) < 5%]. As pointed out by those authors, when protein concentration increases, flow rate decreases and leads to clogging of the filters. Although the methodology described by those authors is a reasonable approach for isolation of hemoglobin, it is calculated that from 30 ml of washed packed cells only about 10 g of hemoglobin can be processed in several hours.

The term lipid-free hemoglobin (LFHb) is preferred to SFHb to clearly distinguish that hemoglobin prepared in the manner described in this paper is essentially free of membrane lipid. Moreover, the term SFHb has been used to describe some hemoglobin preparations which indeed contain membrane lipids. The principle of the process described here has drawn heavily on the techniques employed in work with dialysis encapsulation for preparation of carrier erythrocytes (9–15) and with hollow-fiber dialysis of erythrocytes (16). The most important feature of the process is the controlled dialysis of red blood cells to prevent severe osmotic shock to the cells which results in the complete rupture of the red blood cell membrane and subsequent contamination. Hemoglobin prepared by controlled dialysis and ultrafiltration is almost devoid of lipid and is 99% pure by electrophoretic and HPLC analyses.

METHODS AND MATERIALS

Human blood was obtained from patients with polycythemia vera or hemochromatosis who require periodic bleeding as part of their treatment. It was collected using 63 ml of anticoagulan (citrate phosphate, dextrose, adenine solution) for every 450 ml of blood. Red blood cells were isolated from whole blood by centrifugation at 1000*g* and then washed 3 times in 3 vol of Buffer F (120 mM NaCl, 10 mM glucose, 2 mM $MgCl_2$, 52 mM KH_2PO_4, 18 mM K_2HPO_4). Washed packed cells were adjusted to a 60% (Hct) with Buffer F.

The apparatus used in this procedure consists of a 1-m^2 commercial kidney dialysis hollow-fiber cartridge (Travenol, Oak Park, IL), three 0.1 μm pore hollow-fiber units (1×10^6 molecular weight cutoff, Amicon Corp., Danvers, MD), in the same configuration previously described by DeLoach *et al.* (17). Red blood cells were then dialyzed against 10 liters of 50 mosmol/kg, pH 7.4, dialysis buffer (5.7 mM K_2HPO_4, 2.3 mM KH_2PO_4, 2 mM $MgCl_2$) with a 1-m^2 kidney dialysis hollow-fiber cartridge (Travenol) until an average osmotic pressure of 132 osmol/kg was reached. Flow rates of the cells and dialysis buffer were 200 and 450 ml/min, respectively. The dialyzed red blood cells were then processed through three 0.1 μm pore hollow-fiber units (1×10^6 molecular weight cutoff, Amicon Corp.). The combined surface area of the three ultrafiltration cartridges was 0.09 m^2. During ultrafiltration, dialyzed red blood cells were maintained at a constant volume by replenishing lost volume with a phosphate buffer of the same osmotic pressure as the red blood cells (5 mM KH_2PO_4, 16 mM K_2HPO_4, 34 mM NaCl, 10 mM glucose, 2 mM $MgCl_2$). Osmolarity of the replenishing buffer was adjusted by modifying the amount of NaCl used in the buffer

solution. During ultrafiltration, the flow rate was 200 ml/min and the column pressure was 10–12 psi. All tubing and connectors used were hemo-compatible. The ultrafiltrate was dialyzed against distilled water, lyophilized, and stored at room temperature away from moisture.

For comparison, human hemoglobin was also prepared by a hypotonic dilution technique. Washed packed cells were hemolyzed by diluting to 132 mosmol/kg with ice-cold distilled water. One portion of the hemolysate was centrifuged at 10,000*g* for 30 min, a second portion was filtered through a 0.2 μm pore nylon filter, and the third portion was left untreated. Then all hemolysate preparations were lyophilized and stored at room temperature away from moisture. These preparations were made only for comparison with the LFHb in the following lipid analyses.

Three hundred milligrams of each lyophilized hemoglobin was resuspended in 8 ml of distilled water. The resuspended hemoglobin was slowly added to 13.3 ml of methanol, in a 100 ml beaker, while stirring with a magnetic stir bar. This hemoglobin–methanol mixture was stirred for 30 min. After stirring for 30 min, 27.7 ml of chloroform was slowly added to the methanol–blood mixture and stirred for 2 h. This mixture was then filtered through a coarse porosity sintered glass funnel. The hemoglobin pellet was then homogenized for 1 min in a DuVall tissue homogenizer equipped with a motor driven Teflon pestle. The homogenate was filtered through a coarse porosity sintered glass funnel. The combined filtrates were collected in a 250-ml boiling flask and taken to near dryness on a Buchii rotoevaporator at (30°C). The residue was removed from the flask with five consecutive rinses of $CHCl_3$:MEOH (19:5, v/v) and then filtered through a fine porosity sintered glass funnel. The filtrate was reduced to very near dryness and the residue was dissolved in freshly made $CHCl_3$:MEOH (2:1, v/v), then washed with 0.2 vol of 0.1 M KCl according to the method of Folch (18). The clear chloroform layer was reduced to near dryness for TLC analysis. Lipids were then dissolved in 200 μl of $CHCl_3$:MEOH (19:1, v/v) and streaked on TLC plates.

All solvents used, except diethylether and acetic acid were glass distilled and were purged with argon before use. TLC plates used were coated with silica gel 60 (E Merck, Rahway, NJ). Lipids were separated into classes by developing the TLC plate in a glass tank containing 200 ml of hexane:diethylether:acetic acid (60:40:2, v/v/v). Bands were visualized first with 2-7-dichlorofluorescein and then with iodine vapor. Phospholipids (PL) which remained at the origin were extracted from the silica gel with a mixture of methanol:chloroform (2:1, v/v) followed by extraction with chloroform:methanol (2:1, v/v). The phospholipid extract was streaked onto a TLC plate and developed in a tank containing 200 ml of a chloroform:methanol:ethanol:acetic acid:water (65:25:25:5:4, v/v/v/v/v) mixture. Migration of lipid standards of known composition was used to identify lipid unknowns. Also ninhydrin spray was used to check for phosphatidylserine and phosphatidylethanolamine.

After the PL bands were outlined and the iodine had evaporated from the plates, each band was scraped directly into a 15-ml glass test tube. Adjacent areas of silica corresponding in size and position to the area containing PL were also scraped into test tubes. The silica gel was extracted three times with 2 ml of a mixture of methanol: chloroform (2:1, v/v). The combined extracts were evaporated to dryness and the residues were dissolved in 7 ml of a mixture of chloroform:methanol (19:1, v/v). Aliquots were then analyzed for phosphorus according to the procedures of Duck-Chong (20).

Cholesterol samples were analyzed with a Tracor MT 220 GLC equipped with dual FID and 3% (w/w) OV-17 on a chrom Q (100/120 mesh) column (Supelco, Inc.). Operating conditions were: column temperature, isothermal at 250°C; inlet and detector temperatures at 275 and 300°C, respectively; H_2 and air flow to the detector, 40 and 120 ml/min, respectively; and nitrogen carrier gas at 40 ml/min, for both. Cholesterol in samples was quantitated by comparison of the peak areas with those of known amounts of cholesterol, as computed by a Hewlett–Packard Model 3390A integrator, interphased with the GLC.

LFHb preparations were subjected to SDS–PAGE (21). A 4% stacking gel with a 12.5% running gel was used. Ten microliters of LFHb in sample buffer was loaded in each well on a mini-vertical slab gel apparatus (Bio-Rad, Richmond, CA) at 15 mA constant current for approximately 1 h. After electrophoresis, the gel was stained with Coomassie blue stain.

HPLC analysis was conducted on the LFHb with an instrument equipped with a variable wavelength spectrophotometric detector (LCD/Milton Roy HPLC, Riveria Beach, FL) at 280 nm. A Waters Protein-Pak DEAE 5PW column (7.5 × 75 mm) was used. Protein (10 μl injection) was eluted with a linear NaCl gradient (20 mM Tris, pH 9.2; 0–500 mM NaCl). Spectrophotometric analysis of LFHb samples was conducted from 400 to 600 nm.

The morphology, size, number, and hemoglobin concentration in the red blood cells were monitored throughout the preparative procedure. Cell size, number, and hemoglobin concentration were determined with a Coulter counter equipped with a channel analyzer and a Hemoglobinometer (Coulter Electronics, Hialeah, FL).

For scanning electron microscopy (SEM), cells (0.1 ml) were added to 10 ml of 0.5–1.0% glutaraldehyde prepared in a similar osmotic pressure phosphate buffer. Cells were allowed to fix for 1 h with constant agitation at room temperature. After fixation, cells were washed once in buffer and twice in distilled water and then were dehydrated in 50% ethanol. Cells were then resuspended in 75% ethanol. A drop of this was then transferred to a mounted coverslip. Cells were allowed to air dry and then were coated with gold.

RESULTS

Dialysis of the human red blood cells to an average predetermined osmotic pressure of 132 mosmol/kg (9, 13) allowed the red blood cell membranes to become permeable to hemoglobin without rupture of the membrane. Collection, packing, and washing required 4 h for 3 liters of whole blood. Dialysis requires only 5–20 min with a 1-m^2 hollow-fiber dialysis unit and ultrafiltration requires 10–14 h for 500 ml of packed cells. Dialysis cartridges are used once and discarded. A 10-liter dialysis buffer reservoir can be used to process 1–3 liters of packed cells. Ultrafiltration requires a longer processing time because the protein must pass through the 0.1 μm pore in the hollow fibers. Processing time can be reduced if a larger surface area ultrafiltration cartridge is used. As dialyzed cells are recycled through the hollow fibers, volume is replenished by the use of a closed vessel filled with a pH 7.4 phosphate buffer (5 mM KH_2PO_4, 16 mM K_2HPO_4, 10 mM glucose, 2 mM $MgCl_2$); NaCl concentration was varied to adjust the molarity of buffer to match that of the dialyzed cells.

The data in Table I are the summation of LFHb isolation from three different preparations. In general, 500–600 ml of washed, packed cells can be processed in 9–12 h. Yield of LFHb from the ultrafiltration process averages 90%. Actual recovery

TABLE I

Lipid-Free Hemoglobin Isolation

Volume of packed red blood cells (ml)	*Starting Hb*[a] *g)*	*Ultrafiltration time (h)*	*LFHB recovered (g)*	*% yield*[b]
420	182	11	169.0	92.0
650	183	9	163.0	90.0
420	152	11	135.0	89.0

[a] Hemoglobin.
[b] % yield is calculated as [amount LFHb recovered ÷ (LFHb + Hb remaining in processed cells)] × 100. % yield does not take into account loss of cells due to handling or dead volume in apparatus.

of LFHb may be slightly lower due to loss of cells in handling and because of dead volume in the dialysis and ultrafiltration apparatus. The hematological parameters of red blood cells are recorded in Table II.

SEM photomicrographs of normal, dialyzed and processed cells reveal several interesting features (Fig. 1). Human red blood cells appear as normal biconcave discocytes (Fig. 1A), whereas dialyzed cells appear as uniformly swollen cells (Fig. 1B). Dialyzed cells, unlike red blood cell ghosts, contain normal amounts of hemoglobin and although they are porous, the holes in the membrane are not visible. Random protrusions are observable on some of the dialyzed cells. Once hemoglobin is removed by ultrafiltration, the cells appear mainly as empty ghosts (Fig. 1C). If red blood cells are hemolyzed by direct dilution, cells appear mainly as amorphous stroma (Fig. 1D). It is this form of stroma that can contaminate the hemoglobin preparation.

For comparison and emphasis on the importance of dialysis on the nature of the isolated hemoglobin, red blood cells were hemolyzed by dilution in distilled water and analyzed for the presence of lipids. Phospholipid and neutral lipids were recovered from all hemoglobins prepared by methods other than dialysis–ultrafiltration. The control (lysed red blood cells) contained varying amounts of the individual phospholipids and 656.1 μg of cholesterol. The hemoglobin isolated using the dialysis–ultrafiltration method showed that all samples were essentially free of phospholipids and neutral lipids (Tables III and IV); thus it can be called LFHb.

Such LFHb migrates as a single band on SDS–PAGE (data not shown). HPLC analysis of LFHb yields one major peak with a retention time of 16.34 min. A minor protein peak (probably carbonic anhydrase) followed the elution of LFHb. Integra-

TABLE II

Hematological Parameters[a]

Isolation stage	*g Hb/100 ml*	*Vol (Fl)*
Washed red blood cells	24.3	70.0
Dialyzed cells	24.0	68.7
Processed cells	3.7	28.3

[a] Average of 3 runs. Hb = Hemoglobin.

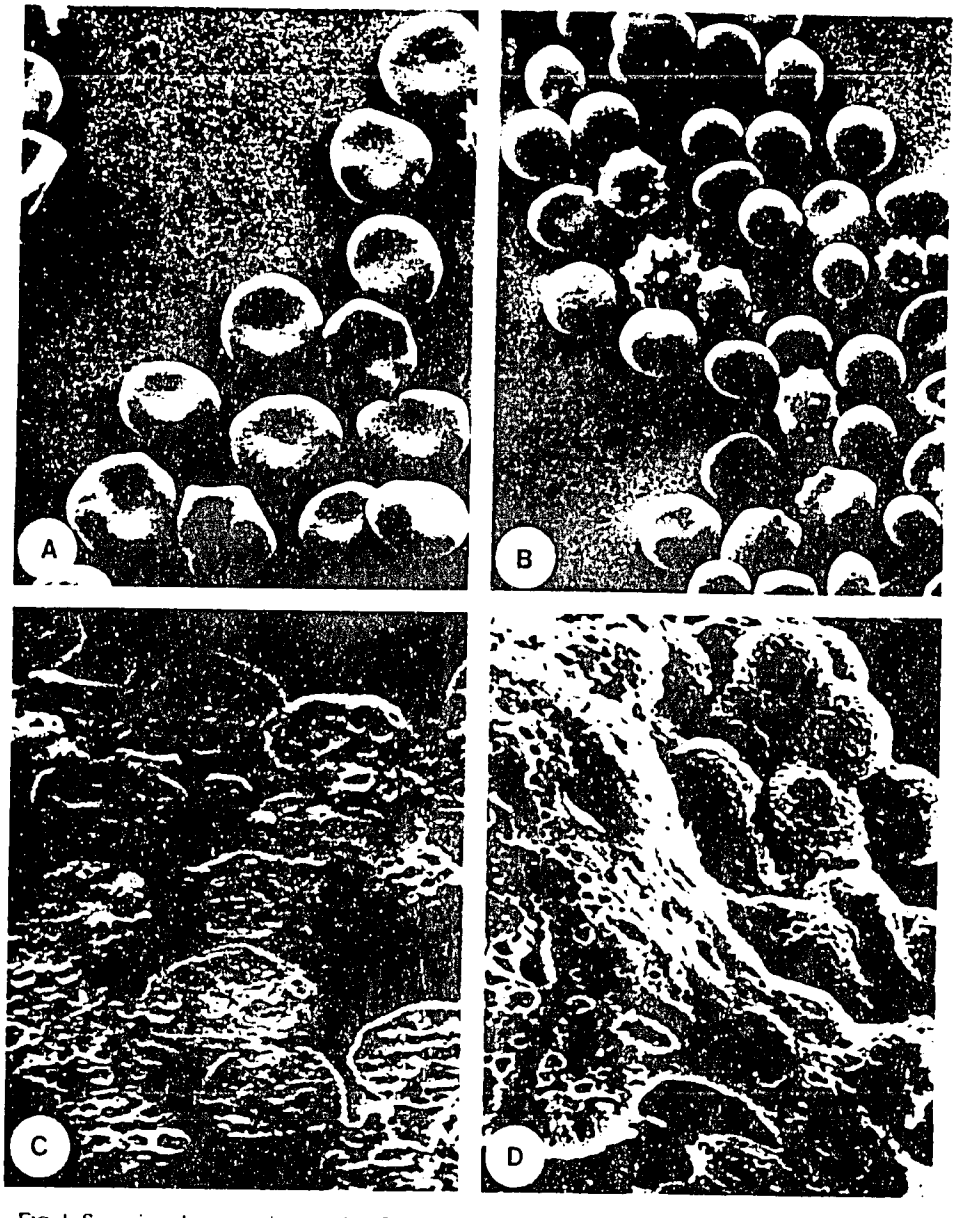

FIG. 1. Scanning electron micrographs of red blood cells. (A) Normal washed human RBC; (B) dialyzed RBC; (C) RBC ghosts; (D) stroma from RBC. A and B, ×2400; C and D, ×3770.

tion of peak areas yields a 99.7% pure hemoglobin sample (Fig. 2). Spectrophotometric analysis between 400 and 600 nm was characteristic of oxyhemoglobin (data not shown).

DISCUSSION

The dialysis–ultrafiltration process outlined here results in the preparation of large volumes of ultrapure lipid-free hemoglobin. The process is extremely dependent on

TABLE III

Phospholipid Content of Human Hemoglobin Preparations

	Method of sample preparation			
Phospholipid	*Control*	*Direct lysis and 0.2 μm filtration*	*Direct lysis and centrifugation*	*Dialysis and ultrafiltration*
SM[a]	13.59	14.10[b]	3.26	0.0[c]
PC	39.70	<0.125	1.45	0.0
PS	14.65	0.000	0.00	0.0
PI	1.57	0.000	0.00	0.0
PE	22.01	0.000	0.00	0.0
PG	5.43	0.000	0.00	0.0
PA	3.29	0.000	0.00	0.0
CL	2.90	0.000	0.00	0.0

[a] SM = sphingomyelin, PS = phosphatidylserine, PC = phosphatidylcholine, PE = phosphatidylethanolamine, PI = phosphaditylinositol, PG = phosphatidylglycerol, PA = phosphatidic acid, CL = cardiolipin.
[b] Data are expressed as μg P_i (inorganic phosphorus)/300 mg of sample.
[c] Sensitivity of phosphorus assay > 0.125 μg P_i.

the way in which the osmotic pressure of the cells is reduced. Reduction of the osmotic pressure of the red blood cells is achieved by controlled hollow-fiber dialysis (16). Red blood cells swell and become permeable to hemoglobin without appreciable cellular lysis. Thus, in the ultrafiltration step, only hemoglobin diffuses through the 0.1 μm pores. If osmotic pressure is reduced by direct dilution in water followed by ultrafiltration, significant lipid contamination is observed. Consequently, advantage is taken of the fact that under controlled dialysis conditions human red blood cells do not appreciably lyse.

The process described here yields a hemoglobin preparation of greater than 99.7% protein purity as evidenced by HPLC analysis. Additionally, the LFHb migrates as a

TABLE IV

Neutral Lipid Content of Human Hemoglobin Preparations

	Method of sample preparation		
Lipid	*Direct lysis and 0.2 μm filtration*	*Direct lysis and centrifugation*	*Dialysis and ultrafiltration*
Monoglycerides	0.00	0.00	0.00
Cholesterol	2.80[a]	24.10	1.80
Diglycerides	0.00	0.00	0.00
Free fatty acids	Trace[b]	0.00	0.00
Fatty acid methyl esters	0.00	0.00	0.00
Triglycerides	0.00	0.00	0.00
Cholesteryl esters	0.00	0.00	0.00

[a] Data are expressed in micrograms/300 mg of sample, and were confirmed by GLC analysis; sensitivity is <100 ng of individual fatty acid and <125 ng cholesterol.
[b] This material could be visualized on the TLC plate using 2,7-dichlorofluorescein and iodine vapor but was below the sensitivity level of the GLC analysis, when separated into individual components.

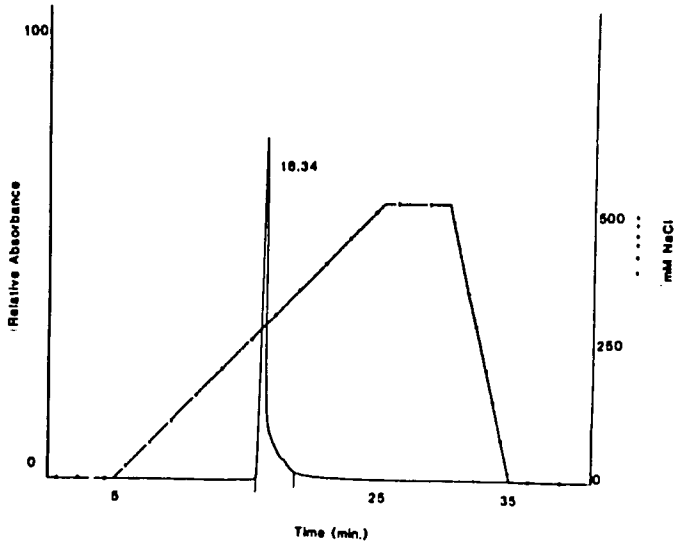

FIG. 2. Human hemoglobin HPLC analysis.

single band on SDS–PAGE. The minor protein contaminant which appears on the DEAE column may be carbonic anhydrase. Although the term SFHb had been applied to different hemoglobin preparations (3, 7), the only evidence for this claim is the lack of an effect on blood coagulation in animals which previously received the hemoglobin preparations. Hemoglobin prepared by dialysis and ultrafiltration contained less than 0.125 μg of P_i, whereas hemoglobin prepared by either direct lysis method contained measurable quantities of P_i migrating with known lipids such as SM and PC. Thus the dialysis and ultrafiltration method is far superior to existing methodologies, based on phospholipid analysis. In addition, neutral lipid analysis revealed appreciable amounts of cholesterol in the direct lysis and centrifugation method. However, only minute quantities of cholesterol were detectable in the hemoglobin prepared by the direct lysis and filtering method or the dialysis and ultrafiltration method. The hemoglobin prepared by direct lysis and filtration contained trace quantities of free fatty acids. Thus, the lipid-free hemoglobin described in this paper contained no detectable quantities of phospholipids, fatty acids, or neutral lipids except for a minute quantity of cholesterol. De Venuto *et al.* (5) presented a crystallization method for SFHb in which no coagulant activity was found, but no lipid analysis was conducted. Others claim that toluene precipitation may lead to irreversible binding of toluene to the hemoglobin (1).

Rosenberry *et al.* (8) reported the use of a filtration technique, but their process required very dilute red blood cell solutions (5% Hct) in order to prevent clogging of the filters. No clogging of hollow fibers was observed with our technique. A considerable improvement in the time required for ultrafiltration is possible if the surface area of the ultrafiltration hollow fibers is increased by using larger cartridges. This process would likely be appropriate for hemoglobin from any mammalian species in which dialysis can be used to open the membrane without cell lysis. For example, dialysis has been used to prepare red blood cells to entrap or encapsulate a variety of exogenous agents without complete rupture of the membrane. Research from this laboratory indicates that human, canine, bovine, ovine, caprine, equine, and murine red

blood cells are sufficiently stable to undergo dialysis (12, 22, 23). However, it is unlikely that the osmotic pressure appropriate for human cells (119–139 mosmol/kg) will be suitable for the red blood cells of other species. It will probably be necessary to use an osmotic pressure close to that which is suitable for the entrapment of exogenous agents (12).

There are several potential applications for LFHb. First, LFHb used for entrapment in liposomes would be more desirable if no contaminating lipids were available for insertion into the lipid vesicles (24). LFHb has potential usefulness in the development of artificial blood diets for blood-feeding arthropods (25–27). This material would be useful for studying the effects of allosteric effectors as well as various toxins and pharmacological agents on the oxygen binding affinity of hemoglobin.

ACKNOWLEDGMENT

The authors thank C. Ayala for technical assistance with the lipid analysis.

RECEIVED: August 26, 1986; REVISED: January 5, 1987

REFERENCES

1. RIGGS, A. (1981) *in* Methods in Enzymology (Antonini, E., Rossi-Berardi, L., and Chiancone, E., Eds.), Vol 76, pp. 5–29, Academic Press, Orlando, FL.
2. SCHOEDER, W. A., AND HUISMAN, T. H. J. (1980) The Chromatograph of Hemoglobins, Dekker, New York.
3. RABINER, S. F., HELBERT, R. J., LOPAS, H., AND FRIEDMAN, L. H. (1967) *J. Exp. Med.* **126**, 1127–1142.
4. DE VENUTO, F., FRIEDMAN, H. I., NEVILLE, J. R., AND PECK, C. C. (1979) *Surg. Gynecol.* **149**, 417–437.
5. DE VENUTO, F., ZUCK, T. F., ZEGNA, A. I., AND MOORES, W. Y. (1977) *J. Lab. Clin. Med.* **89**, 509–516.
6. CHEUNG, L. C., STORM, C. B., GABRIEL, B. W., AND ANDERSON, W. A. (1984) *Anal. Biochem.* **137**, 481–484.
7. RABINER, S. F., O'BRIEN, K., PESKIN, G. W., AND FRIEDMAN, L. H. (1970) *Ann. Surg.* **171**, 615–622.
8. ROSENBERRY, T. L., CHEN, J. F., LEE, M. M. L., MOULTON, T. A., AND ONIGMAN, P. (1981) *J. Biochem. Biophys. Methods* **4**, 39–38.
9. DELOACH, J. R., HARRIS, R. L., AND IHLER, G. M. (1980) *Anal. Biochem.* **102**, 220–227.
10. DELOACH, J. R., CULLER, K., AND HARRIS, C. L. (1980) *J. Appl. Biochem.* **2**, 177–182.
11. DELOACH, J. R., AND BARTON, C. (1981) *Amer. J. Vet. Res.* **2**, 667–669.
12. DELOACH, J. R., AND SPRANDEL, U. (eds) (1985) Red Blood Cells as Carriers for Drugs, Bibliotheca Haematologica Vol. 51, Krager, Basel.
13. DELOACH, J. R., *et al.* (1982) U.S. patent 4,327,710.
14. DELOACH, J. R., *et al.* (1983) U.S. patent 4,389,209.
15. DELOACH, J. R., *et al.* (1985) U.S. patent 4,517,080.
16. DELOACH, J. R. (1985) *Amer. J. Vet. Res.* **46**, 1089–1091.
17. DELOACH, J. R., SHEFFIELD, C. L., AND SPATES, G. E. (1986) *Anal. Biochem.* **157**, 191–198.
18. FOLCH, J., LEES, M., AND STANLEY, G. H. (1957) *J. Biol. Chem.* **226**, 497–504.
19. SPATES, G. E., AND DELOACH, J. R. (1986) *Comp. Biochem. Physiol. A* **83**, 667–671.
20. DUCK-CHONG, C. G. (1978) *Lipids* **14**, 497–499.
21. LEAMMLI, U. K. (1970) *Nature (London)* **227**, 680–685.
22. DELOACH, J. R., AND DROLESKEY, R. (1986) *Comp. Biochem. Physiol. A* **83**, 772–777.
23. DELOACH, J. R., AND DROLESKEY, R. (1986) *Amer. J. Vet. Res.* **47**, 2368–2372.
24. HUNT, C. A., BURNETTE, R. R., MCGREGOR, R. D., *et al.* (1985) *Science* **1165**, 1980–1982.
25. DELOACH, J. R., AND TAHER, M. (1982) *Bull. Ent. Res.* **663**, 72–74.
26. DELOACH, J. R., AND SPATES, G. (1984) *Southwest Entomol.* **9**, 28–32.
27. KABAYO, J., TAHER, M., AND VAN DER FLOEDT, A. (1985) *Bull. Ent. Res.* **75**, 635–640.

2. H- 64

BIOMAT., ART. CELLS, ART. ORG., 16(5), 887-904 (1988-89)

HYPOOSMOTIC DIALYSIS AND ULTRAFILTRATION TECHNIQUE FOR PREPARATION OF MAMMALIAN HEMOGLOBIN: A COMPARISON OF THREE SPECIES.

C.L. Sheffield, G.E. Spates, and J.R. DeLoach
Veterinary Toxicology and Entomology Research Laboratory
Agricultural Research Service,
United States Department of Agriculture
College Station, Texas, 77841

Bustio Sol. E.

ABSTRACT

Dialysis of human, bovine, and ovine red blood cells using a hypotonic solution and a commercial kidney dialysis unit, followed by ultrafiltration through 0.1μm pore hollow-fibers provides an easily managed method for isolation of lipid-free hemoglobin (LFHB). High performance liquid chromatography (HPLC) analysis of hemoglobin indicated 99% protein purity. SDS polyacrylamide gel electrophoresis analysis demonstrated that LFHB migrates as a single band. Processing one-half liter of packed red blood cells requires approximately 10 hours and resulting in an average of 93% hemoglobin recovery.

INTRODUCTION

Hemoglobin accounts for more than 95% of the soluble protein in red blood cells and is one of the most important proteins found in mammals. Its main biological function is the transport

Copyright © 1989 by Marcel Dekker, Inc.

Rec'd in SCI APR 10 1990

of respiratory gases within the tissues of the body. A substantial amount of research has been done on hemoglobin [1-7]. One area of hemoglobin research that has received substantial attention is the evaluation of hemoglobin as a possible blood substitute [3-8]. A major obstacle in this research is the lack of a simple, inexpensive and fast method of preparing large quantities of ultrapure hemoglobin suitable for use as a blood substitute.

Currently hemoglobin isolation is performed using one of the following techniques. Riggs [6] suggests lysing the red blood cells with water and removing membrane stroma by centrifugation. De Venuto et al. [7] proposes hemolysis with water and toluene, low speed centrifugation, and crystallization using a 2.8M sodium phosphate buffer. However, such treatment with organic solvents sometimes denatures the hemoglobin [9]. Cheung et al. [8] prepares stroma-free hemoglobin by selective DEAE-cellulose absorption. This method still involves hemolysis of the red blood cell membrane which can result in lipid contamination of the hemoglobin.

This paper describes a technique for the rapid large scale isolation of ultrapure essentially lipid-free hemoglobin (LFHb) from several mammalian species. The most important feature of the process is the controlled dialysis of red blood cells. This process is similar to the techniques employed in work with dialysis encapsulation for the preparation of carrier erythrocytes [10-13]. Controlled dialysis prevents severe osmotic shock which results in the rupture of the red blood cell membrane and subsequent contamination [14,15].

This technique results in the isolation of a hemoglobin product that (1) migrates as a single band on SDS-PAGE, (2) is 99% pure by HPLC analysis, and (3) shows essentially no lipid contamination upon analysis. An average hemoglobin recovery of 93% can be obtained with an ultrafiltration processing time of approximately 10 hours.

MATERIALS AND METHODS

Preparation of Lipid-free Hemoglobin

Human blood was obtained from Dr. Ralph Green of the Cleveland Clinic Foundation, Cleveland Ohio. Bovine and ovine blood were collected in 2-liter evacuated containers, using heparine (Sigma, St. Louis, Mo) as the anticoagulant (0.1mg/ml). Red blood cells were isolated from whole blood by centrifugation at 1000 g and then washed 3-5 times in 3 volumes of either phosphate buffer solution (PBS- 120 mM NaCl, 10 mM glucose, 2mM $MgCl_2$, 70 mM potassium phosphate, pH 7.2) or 0.9% NaCl solution. Washed packed cells were adjusted to a 60% (Hct) with PBS or 0.9% NaCl. The apparatus used in this procedure was described in our previous publications [14,15]. Red blood cells were dialyzed at 4°C against 10-18 liters of dialysis buffer (50 mOsm/Kg, pH 7.4 or 100 mOsm/Kg, pH 7.4) with a 1 m^2 kidney dialysis hollow-fiber cartridge (Travenol, Oak Park, IL). Dialysis continued until an average osmotic pressure of 132 Osm/Kg (human), 170 Osm/Kg (bovine), or 204 Osm/Kg (ovine) was reached. Flow rates of the cells and dialysis buffer were 200 and 450 ml/min, respectively. The dialyzed red blood cells were then processed at 4°C through three 0.1 μm pore hollow-fiber units (1 X 10^6 molecular weight cut off, Amicon, Danvers, MA.) The combined surface area of the three ultrafiltration cartridges was 0.09 m^2. During ultrafiltration, dialyzed red blood cells were maintained at a constant volume by replenishing lost volume with a phosphate buffer (21 mM potassium phosphate, pH 7.2, 10 mM glucose, 2mM $MgCl_2$) of the same osmotic pressure as the red blood cells. Osmotic pressure of the replenishing buffer was adjusted by varying the amount of NaCl used in the buffer solution. During ultrafiltration, the flow rate of the cells was 200 ml/min and 10-12 psi column pressure. All tubing and connectors used were hemo-compatible. The ultrafiltrate was lyophilized and stored at room temperature away from moisture.

For comparison, hemoglobin was also prepared by a hypotonic dilution technique. Washed packed cells were hemolyzed by diluting 1:4 with ice cold distilled water. One portion of the hemolysate was centrifuged at 10,000 g for 30 min, a second portion was centrifuged at 10,000 g for 30 min and then filtered through a 0.2 micron pore nylon filter, and the third portion was left untreated. Then all hemolysate preparations were lyophilized and stored at room temperature away from moisture. These preparations were made only for comparison with the LFHb in the following lipid analyses.

Lipid Analysis

A sample of each lyophilized hemoglobin was resuspended in 2 ml of distilled water at 10-12 g%. Round bottom flasks (100 ml) containing hemoglobin were placed in an ice bath and 20 ml of ice cold methanol were slowly added while stirring with a magnetic stir bar. The contents of the flask were filtered through silanized glass wool. The filtrate was collected in a 100 ml flask and immediately roto-evaporated to dryness. A small amount of benzene and ethanol was added to the flask to prevent bumping and to remove final traces of water. The dried lipid was then dissolved in 5 ml of chloroform:methanol (19:1, v/v). Extraction of the lipid from the flask was repeated three times. Each rinse solution was then filtered through medium porosity (10-15 μm) sintered glass filter. The filtrate was reduced to dryness under argon gas. Samples were then subjected to a Folch wash [16], and concentrated for TLC and other analysis Spates [17].

For subsequent analysis of lipids, all solvents except diethylether and acetic acid were glass distilled and all solvents were purged with argon before use. Finally, all procedures were carried out under an argon atmosphere. TLC plates used were coated with Silica gel 60, (E Merck, Rahway, NJ). To separate lipids into classes, a hexane:diethylether :acetic acid (60:40:2, v/v/v) solvent system was used. Lipids

were visualized by iodine vapor. Phospholipids (PL) which remained at the origin were further chromatographed by developing the plate in the same direction with a cholorform:methanol :ethanol:acetic acid:water (65:25: 25:5:4, v/v/v/v/v) solvent system. Migration of lipid standards of known composition was used to identify lipid unknowns.

After the PL bands were outlined and the iodine had evaporated from the plates, each band was scraped directly into a 15-ml glass test tube. Adjacent areas of silica corresponding in size and position to the area containing PL were also scraped into test tubes. The silica gel was extracted three times with 2 ml of a mixture of methanol-chloroform (2:1, v/v). The combined extracts were evaporated to dryness and the residues were dissolved in 7 ml of a mixture of chloroform-methanol (19:1, v/v). Aliquots were then analyzed for phosphorus according to procedures of Duck-Chong [18].

Cholesterol samples were analyzed with a Tracor MT 220 GLC equipped with dual FID and 3% (w/w) OV-17 on chrom Q (100/120 mesh) column (Supelco, Inc., Bellefonte, PA). Operating conditions were: column temperature, isothermal at 250°C; inlet and detector temperatures at 275°C and 300°C, respectively; H_2 and air flow to the detector, 40 and 120 ml/min, respectively; and nitrogen carrier gas at 40 ml/min, for both. Cholesterol was quantitated by comparison of the peak areas with those of known amounts of cholesterol, as computed by a Hewlett Packard model 3390A integrator, interfaced with the GLC.

As a further check for the presence of lipid, 100 μg of hemoglobin from each sample was subjected to methanol-NaOH hydrolysis according to the method of Kates [19]. Saponifiable and non-saponifiable lipids were analyzed on a Shimadzu capillary gas chromatograph model GC-9A (Kyoto,Japan).

Electrophoretic and HPLC analysis

LFHb preparations were subjected to SDS-PAGE Leammli [20]. A 4% stacking gel with a 12.5% running gel was used. Each well was

loaded with 10 μl of LFHb in sample buffer and run on a mini-vertical slab gel apparatus (Bio-Rad, Richmond, CA) at 15 mA constant current for approximately 1 h. After electrophoresis, the gel was stained with Coomassie Brilliant Blue.

HPLC analysis was conducted on the LFHb with an instrument equipped with a variable wavelength spectrophotometric detector (LCD/Milton Roy, Riviera Beach, FL) at 280 nm. A Waters Protein-Pak DEAE 5PW column (7.5 X 75 mm) was used. Protein (10 μl/injection) was eluted with a linear NaCl gradient (20 mM Tris, pH 9.2; 0-500 mM NaCl). Absorbance spectrum of LFHb samples was conducted between 400 - 600 nm.

Hematological Analysis

The morphology, size, number, and hemoglobin concentration in the red blood cells were monitored throughout the preparative procedure. Cell size and number were determined using a Coulter Counter equipped with a channel analyzer. Hemoglobin concentration was determined using a Coulter Hemoglobinometer (Coulter Electronics, Hialeah, FL).

Scanning Electron Microscopy

For scanning electron microscopy (SEM) cells (0.1 ml) were added to 10 ml of 0.5-1.0% glutaraldehyde prepared in a similar osmotic pressure phosphate buffer. Cells were allowed to fix for 1 h with constant agitation at room temperature. After fixation, cells were washed once in buffer, twice in distilled water, then dehydrated in 50% ethanol. Cells were then resuspended in 75% ethanol. A drop of this was then transferred to a mounted coverslip. Cells were allowed to air dry, then coated with gold.

RESULTS

Collection, packing, and washing required 4 h for 3 liters of whole blood. Dialysis of the mammalian red blood cells to an

TABLE I
Lipid-free Hemoglobin Isolation

Vol. of packed red blood cells (ml)	Total Hb[a] (g)	Ultrafiltration Time (h)	LFHb recovered (g)	Yield[b] (%)
Bovine				
551	112	5	107	95
Human				
497	172	10	156	90
Ovine				
643	136	15	131	94

[a]Total Hb = (LFHb + Hb remaining in processed cells)
[b]% yield is corrected for the loss of cells due to handling or dead volume in apparatus.
Data are the mean of three isolations for each species.

average predetermined osmotic pressure of 132-204 mOsm/Kg allowed the red blood cells membrane to become permeable to hemoglobin without rupture of the membrane [13-15]. Dialysis required only 5-20 minutes with a 1 m^2 hollow fiber dialysis unit and ultrafiltration required 6-14 h for 500 ml of packed cells. Kidney dialysis cartridges were used once and discarded. An 11 liter dialysis buffer reservoir can be used to process 1-3 liters of packed cells. Ultrafiltration required a longer processing time because the protein must pass through the 0.1 μ pore in the hollow fibers. Ultrafiltration time can be reduced if a larger surface area ultrafiltration cartridge is used. As dialyzed cells were recycled through the hollow fibers, volume was replenished by the use of a closed vessel filled with replenishing buffer of the same osmolarity as the cells.

Average LFHb isolation values from each of the species are summarized in Table I. In general, three liters of whole blood can be completely processed in 15-20 h. Yield of LFHb from the ultrafiltration process averaged 93%. Actual recovery may be slightly lower due to loss of cells in handling and because of dead volume in the dialysis and ultrafiltration apparatus. The hematological values for the red blood cells are recorded in Table II.

TABLE II
Hematological Parameters

Species & Parameter	Isolation Stages		
	Washed RBC	Dialyzed Cells	Processed Cells
Bovine			
Hb (ng/cell)	1.5[a]	1.5	0.1
Volume (fl)	47.1	58.0	27.5
Human			
Hb (ng/cell)	2.4[b]	2.4	0.4
Volume (fl)	70.0	68.7	28.3
Ovine			
Hb (ng/cell)	1.2[b]	1.0	0.2
Volume (fl)	32.8	39.8	17.0

[a]Data are the mean of four experiments.
[b]Data are the mean of three experiments.
fl = Femtoliter, ng = Nanograms

Scanning Electron photomicrographs of normal, dialyzed and processed cells, from each species revealed several interesting features (Fig. 1-3). Washed red blood cells appeared as normal biconcave diskocytes (Fig. 1), whereas dialyzed cells appeared as uniformly swollen cells (Fig. 2). Dialyzed cells, unlike red blood cell ghosts, contain normal amounts of hemoglobin and although they are porous, the holes in the membrane are not visible. Random protrusions were observable on some of the dialyzed cells. Once hemoglobin is removed by ultrafiltration, the cells appeared mainly as empty ghosts (Fig. 3a-c). If red blood cells are hemolyzed by direct dilution, cells appeared mainly as amorphous stroma (Fig. 3d). It is this form of stroma that can contaminate the hemoglobin preparation.

For comparison and emphasis on the importance of dialysis on the nature of the isolated hemoglobin, red blood cells were hemolyzed by dilution in distilled water and analyzed for the presence of lipids. The hemoglobin isolated using the dialysis-ultrafiltration method showed that all samples were essentially free of phospholipids and cholesterol, thus it can be called LFHb (Table III).

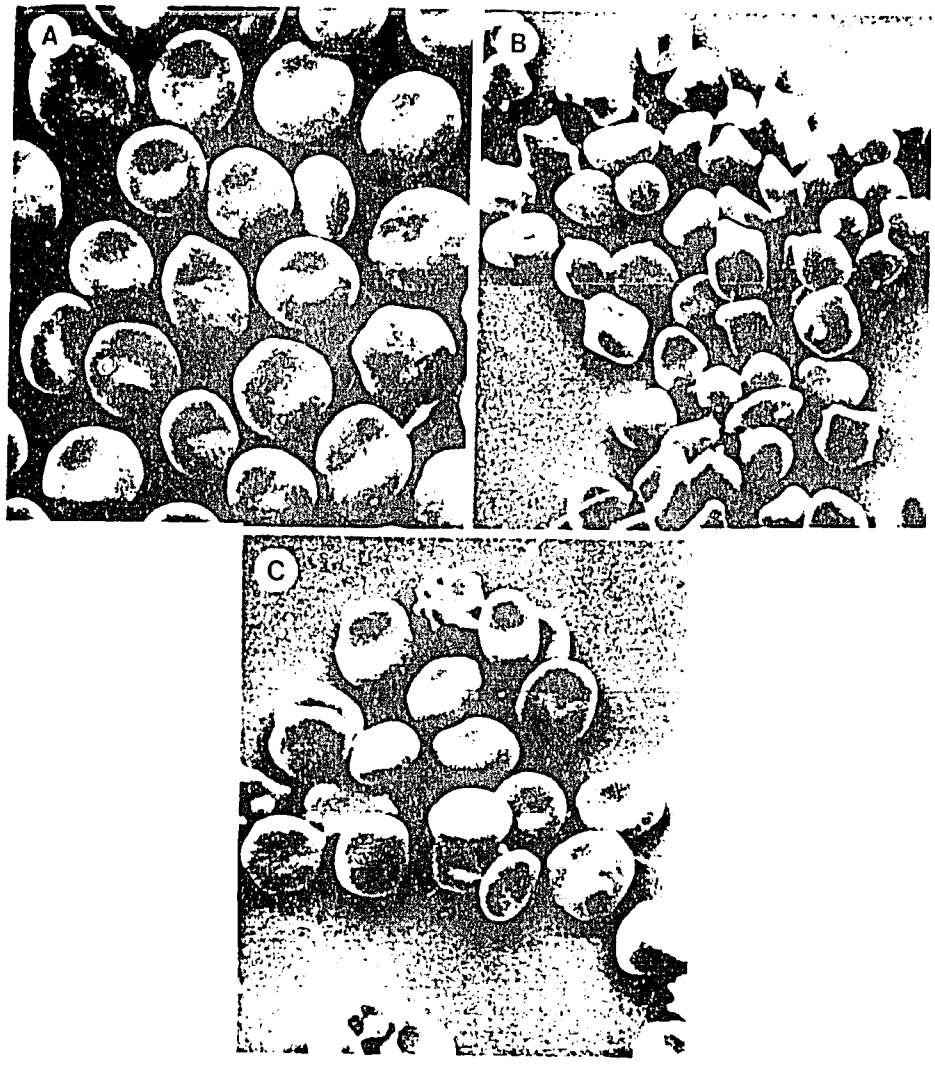

Fig. 1. Scanning electron micrographs of normal washed red blood cells a) bovine, b) ovine, c) human

29

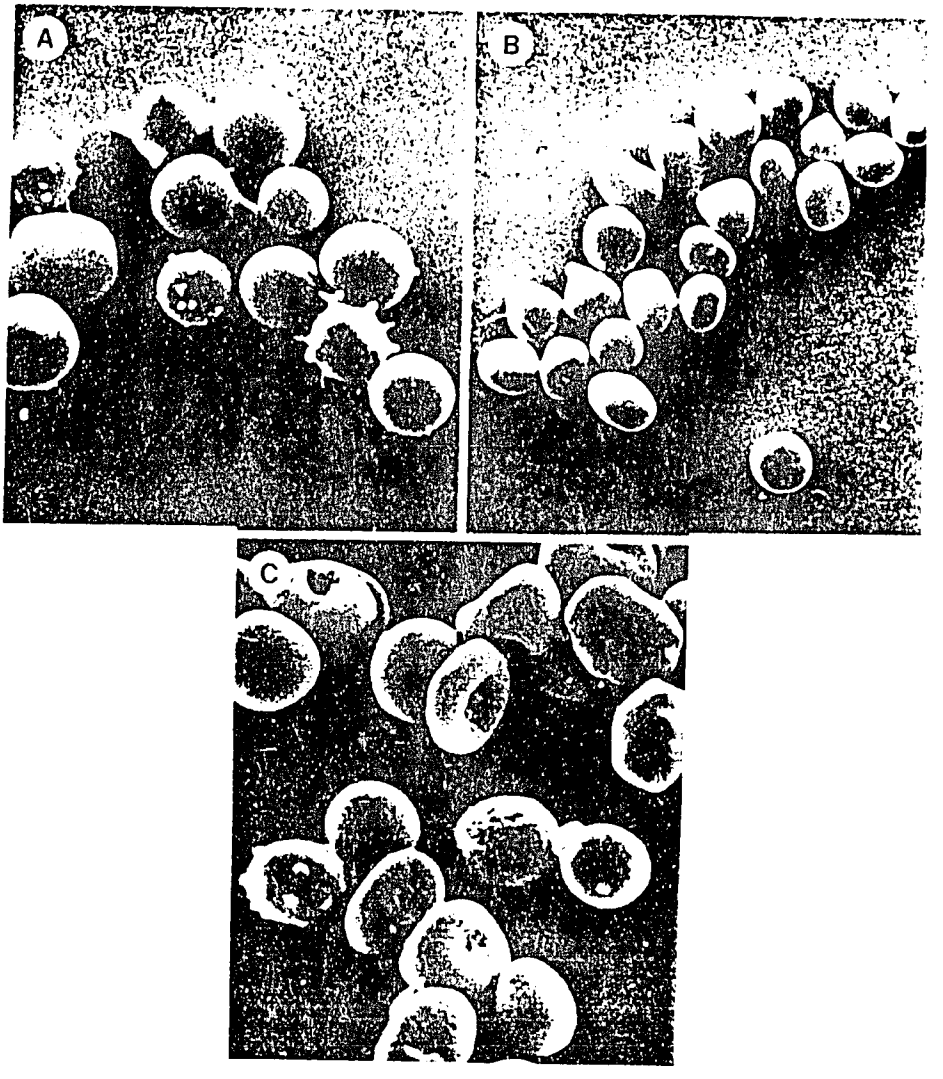

Fig. 2. Scanning electron micrographs of dialyzed red blood cells a) bovine, b) ovine, c) human

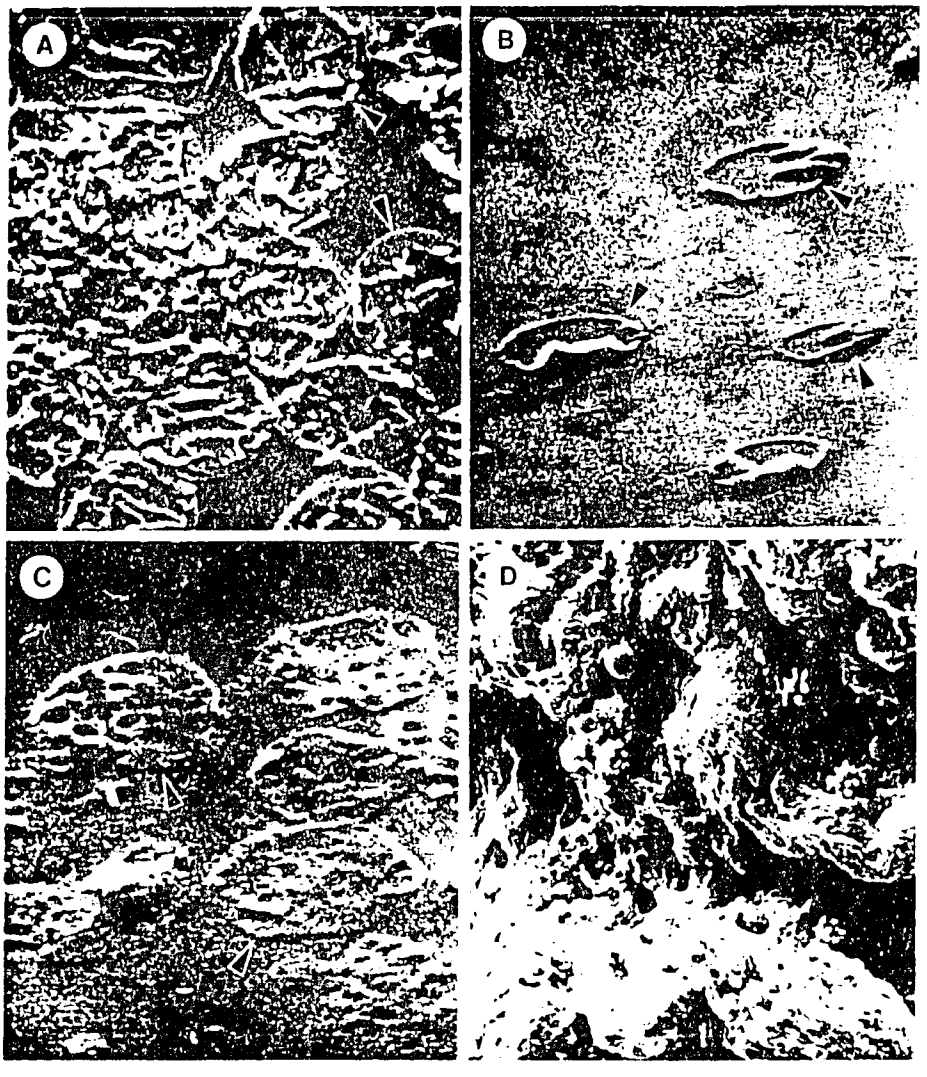

Fig. 3. Scanning electron micrographs of RBC-ghosts a) bovine, b) ovine, c) human, d) amorphous stroma

TABLE III
Lipid Contents of Hemoglobin Preparations

Species & Lipid Classes	Method of sample Preparation A	B	C
Bovine			
Cholesterol	2190.000[a]	660.000	0.000[b]
SM[c]	0.047[d]	0.011	0.000
PS	0.006	0.003	0.000
PE	0.011	0.006	0.000
PC	0.000	0.000	0.000
Human			
Cholesterol	2.800	24.100	1.800
SM	14.100	3.260	0.000
PS	0.000	0.000	0.000
PE	0.000	0.000	0.000
PC	<0.125	1.450	0.000
Ovine			
Cholesterol	57.200	472.000	0.950
SM	2.530	14.470	0.420
PS	0.030	0.850	0.000
PE	1.310	3.930	0.420
PC	0.000	0.000	0.000

[a]Data are the mean and are expressed in micrograms/300 mg of sample.
[b]There were no detectable lipids from the TLC plate.
[c]SM - sphingomyelin, PS - phosphatidyl serine, PE - phosphatidyl ethanolamine, PC - phosphatidyl choline.
[d]Data are expressed in micrograms P_i(inorganic phosphorus)/300 mg of sample.

Analysis of LFHb by HPLC yields one major peak followed by a minor protein peak, probably carbonic anhydrase. Integration of peak areas yields a 99.7% pure hemoglobin sample (Fig. 4). Such LFHb migrates as a single band on SDS-PAGE (data not shown). The visible spectra analysis between 400-600 nm was characteristic of oxyhemoglobin (Fig. 5)

DISCUSSION

The RBC of cattle, sheep and humans all have a similar appearance, however they are quite different with respect to size, volume and most importantly chemical composition of their membrane.

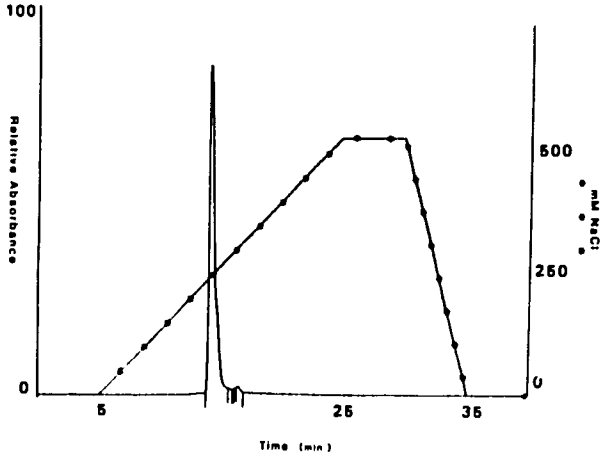

Fig. 4. Typical hemoglobin HPLC analysis peak

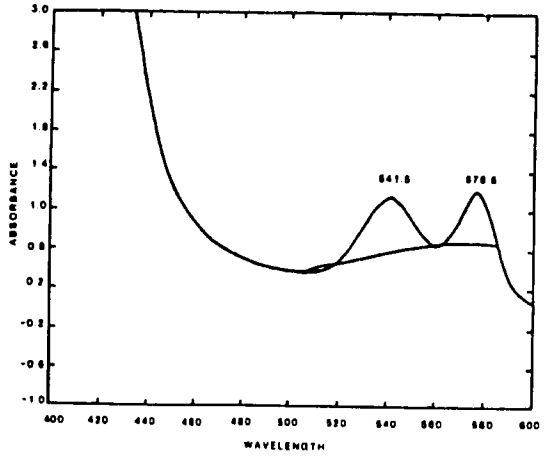

Fig. 5. Visible spectral scan of hemoglobin

For example, human RBC have an average diameter of 8.5 μM and an average volume of 87 fl, in cattle these values are 5.8 μM and 52 fl respectively, while in sheep the average diameter of a RBC is only 4.5 μM with a volume of 35 fl [21].

The RBC of these three species have quite different osmotic fragilities and membrane phospholipid compositions. In humans initial hemolysis occurs at a NaCl concentration of 0.44% [22], in cattle and sheep initial hemolysis occurs at 0.59% and 0.67% respectively [21].

Another area of major difference is the phospholipid composition of the RBC membrane found in the three species. Cattle and sheep RBC membranes do not contain phosphatidyl choline (PC), however in human RBC PC accounts for almost 29% of the total phospholipids in the membrane. These three species of RBC also differ in the overall proportions of phospholipids their membranes contain. For example, in humans the three main phospholipids in the RBC membrane are PC 29%, phosphatidyl ethanolamine (PE) 27% and sphingomyelin (SM) 27%. The remainder of the phospholipids include phosphatidyl serine (PS) 13%, phosphatidyl inositol 2% and phosphatidic acid (PA) 2%. However, in cattle and sheep SM is the most predominate phospholipid in the RBC membrane accounting for 46% (cattle) and 51% (sheep) of the total phospholipid content of the membrane. Phosphatidyl choline is the second most abundant RBC membrane phospholipid in both cattle and sheep, accounting for 29% and 26% respectively. The third main RBC membrane phospholipid is PS with concentrations of 19% in cattle and 14% in sheep. The last two identifiable phospholipids in cattle and sheep RBC membranes are PI 4% in cattle and 3% in sheep and PA which is less than 0.3% in both species [23].

In spite of the physical and chemical differences between human, cattle and sheep RBC, the technique described here is applicable to all three. We believe that this technique will work for any species of RBC which is suitable for encapsulation.

The dialysis, ultrafiltration process outlined here results in the preparation of large volumes of ultrapure essentially

lipid-free hemoglobin. The process is extremely dependent on the method used to reduce the osmotic pressure of the cells. Reduction of the osmotic pressure of the red blood cells is achieved by controlled hollow-fiber dialysis [13]. Red blood cells swell and become permeable to hemoglobin without appreciable cellular lysis. Thus, in the ultrafiltration step, only hemoglobin diffuses through the 0.1 μm pores. If osmotic pressure is reduced by direct dilution in water followed by ultrafiltration, significant lipid contamination is observed.

The process described here yields a hemoglobin preparation of greater than 99.7% protein purity as evidenced by HPLC analysis. The minor protein contaminant which appears on the DEAE column may be carbonic anhydrase. Additionally, the LFHb migrates as a single band on SDS-PAGE. Although the term SFHb had been applied to different hemoglobin preparations [3,5,8] the only evidence for this claim is the lack of enhanced clotting times for animals which previously received the hemoglobin preparations. De Venuto et al. [7] presented a crystallization method for SFHb in which no coagulant activity was found but no lipid analysis was conducted. Others claim that toluene precipitation may lead to irreversible binding of toluene to the hemoglobin [6].

Rosenberry et al. [24] reported the use of a filtration technique, but their process required very dilute red blood cell solutions (5% Hct) in order to prevent clogging of the filters. No clogging of hollow-fibers was observed with our technique. A considerable improvement in the time required for ultrafiltration is possible if the surface area of the ultrafiltration hollow-fibers is increased by using larger cartridges. This process would likely be appropriate for hemoglobin from any mammalian species in which dialysis can be used to open the membrane without cell lysis. Research from this laboratory indicates that human, canine, porcine, bovine, ovine, caprine, equine, and murine red blood cells are sufficiently stable to undergo dialysis [12,13,25]. It will probably be necessary to use an osmotic pressure close to that which is suitable for the entrapment of exogenous agents [12,13,25].

There are several potential applications for LFHb. It could be entrapped in liposomes, which would then be used for transfusions. LFHb would be more desirable for entrapment since it contains fewer contaminating lipids which could be inserted into the lipid vesicles [26]. This material would also be valuable in studying the effects of various toxins and pharmacological agents on the oxygen binding affinity of hemoglobin. LFHb is also potentially useful in the development of artificial diets for blood-feeding arthropods [2,17].

ACKNOWLEDGMENTS

The authors thank R. E. Droleskey for preparation of the Scanning Electron Micrographs, and for all photographic processing of micrographs and figures. We also thank E. Moore for assistance with preparation of Scanning Electron Micrographs, and C. Ayala for technical assistance with the lipid analysis.

REFERENCES

1. J.R. Neville, Hemoglobin oxygen affinity measurement using biotonometry., J. Appl. Phys., 37, 967, (1974).

2. J. Kabayo, M. Taher and A. Van der Floedt, Development of a Synthetic Diet for Glossina (Diptera: Glossinidea), Bull. Ent. Res., 75, 635, (1985).

3. S.F. Rabiner, Hemoglobin solution as a plasma expander, Fed. Proc., 34, 1454, (1975).

4. W. Mok, D. Chen, and A. Mazur, Cross-linked hemoglobin as potential plasma expanders, Fed. Proc., 34, 1458, (1975).

5. H.R. Kaplan and V.S. Murthy, Hemoglobin solution : a potential oxygen transporting plasma expander. Fed. Proc. 34, 1461, (1975).

6. A. Riggs, Methods in Enzymology, 76, 5, (1981).

7. F. De Venuto, T.F. Zuck, A.I. Zegna, and W.Y. Moores, Characteristics of Stroma-free Hemoglobin Prepared by Crystallization, J. Lab. Clin. Med., 89, 509, (1977).

8. L.C. Cheung, C.B. Storm, B.W. Gabriel, and W.A. Anderson, The Preparation of Stroma-free Hemoglobin by Selective DEAE-cellulose Absorption, Anal. Biochem., 137, 481,(1984).

9. W.A. Schoeder and T.H.J. Huisman, The Chromatograph of Hemoglobin's, Dekker, New York, (1980).

10. J.R. DeLoach, R.L. Harris, and G.M. Ihler, An Erythrocyte Encapsulator Dialyzer Used in Preparing Large Quantities of Erythrocyte Ghosts and Encapsulation of a Pesticide in Erythrocyte Ghosts, Anal. Biochem., 102, 220, (1980).

11. J.R. DeLoach, R.L. Harris, G.M. Ihler, and R.T. Mayer, U.S. Patent 4,517,080, (1985).

12. J.R. DeLoach and U. Sprandel (eds), Red Blood Cells as Carriers for drugs, Bibliotheca Haematologica, 51, Karger, Basel.(1985)

13. J.R. DeLoach, Continuous-flow Hollow-fiber Dialysis System for Preparation of Bovine Carrier Erythrocytes, Amer. J. Vet. Res., 46, 1089, (1985).

14. J.R. DeLoach, C.L. Sheffield, and G.E. Spates, A Continuous -flow High-yield Process for Preparation of Lipid-free Hemoglobin, Anal. Biochem., 157, 191, (1986).

15. C.L. Sheffield, G.E. Spates, R.E. Droleskey, R. Green, and J.R. DeLoach, Preparation of Lipid-free Human Hemoglobin by Dialysis and Ultrafiltration, Biotechnology and Applied Biochemistry, 9, 230, (1987).

16. J. Folch, M. Lees, and G.H. Stanley, A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, J. Biol. Chem., 226, 497, (1957).

17. G.E. Spates and J.R. DeLoach, Possible Correlations Between Reproductive Performance of Stomoxys calcitrans and Lipid Content of Blood Diets, Comp. Biochem. Physical., 83A, 667, (1986).

18. C.G. Duck-Chong, A Rapid Sensitive Method for Determing Phospholipid Phosphorous Involving Digestion with Magnesium Nitrate, Lipids, 14, 497, (1978).

19. M. Kates, Isolation, analysis and identification of lipids, Techniques of Lipidology, North-Holland Publishing Comp., Amsterdam, (1975).

20. U.K. Leammli, Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4, Nature, 227, 680, (1970).

31

21. R.B. Thompson and S.J. Proctor, A Concise Textbook of Hematology, 6th Ed., Urban & Schwarzenberg Inc., Baltimore Maryland, 1984.

22. John B. Maile, Laboratory Medicine : Hematology, 4th Ed., C.V. Mosby Comp., St.Louis, MO, 1972.

23. G. Rouser, G.J. Nelson, S. Fleischer, and G. Simon, Biological Membranes: Physical Fact and Function, Chapter 2, Academic Press, London and New York, (1968).

24. T.L. Rosenberry, J.F. Chen, M.M.L. Lee, T.A. Moulton, and P. Onigman, Large Scale Isolation of Human Erythrocyte Mem branes by High Volume Molecular Filtration, Biochem. Biophys. Methods, 4, 38, (1981).

25. J.R. DeLoach and R. Droleskey, Preparation of Ovine Carrier Erythrocytes: Their Action and Survival, Comp. Biochem. Physical, 83A, 772, (1986a).

26. C.A. Hunt, R.R. Burnette, R.D. McGregor, et al., Synthesis and Evaluation of a Prototype Artificial Red Cell, Science, 1165, 1980, (1985).

ANALYTICAL BIOCHEMISTRY 157, 191–198 (1986)

2. H-04

A Continuous-Flow High-Yield Process for Preparation of Lipid-Free Hemoglobin[1]

JOHN R. DELOACH, CYNTHIA L. SHEFFIELD, AND GEORGE E. SPATES

Veterinary Toxicology Research Unit, Veterinary Toxicology and Entomology Research Laboratory, Agricultural Research Service, United States Department of Agriculture, College Station, Texas 77841

Received January 21, 1986

Hypotonic hollow-fiber dialysis of bovine red blood cells followed by ultrafiltration through 0.1-μm pore hollow fibers provides a simple method for isolation of lipid-free hemoglobin. Hemoglobin (Hb) isolated by comparative techniques were all contaminated with membrane stroma. HPLC analysis of Hb revealed a protein peak of 99.6% purity and sodium dodecylsulfate–polyacrylamide gel electrophoresis analysis revealed a single band. The process requires hypoosmotic dialysis of bovine RBC to a final 160–180 mosmol/kg osmotic pressure. Additional reduction in osmotic pressure causes irreversible cell lysis which leads to lipid contamination of the Hb. Processing of ½ liter of packed red blood cells requires 4–5 h, resulting in an average of 90% hemoglobin recovery. © 1986 Academic Press, Inc.

Hemoglobin is the major protein constituent of red blood cells (RBC)[2] and comprises over 95% of the total soluble protein of the cell. Isolation of hemoglobin (Hb) from RBC involves a simple procedure of hemolyzing the RBC followed by centrifugation to presumably remove membrane stroma. Hemolysis results in a Hb solution that is more than 90% pure (1). In some cases organic solvents such as toluene have been used as hemolytic agents (2). However, such treatment often denatures the Hb.

Although stroma-free Hb (SFHb) has been evaluated as a blood substitute (3,4), there exists no methodology to prepare large quantities of SFHb. Crystallization from a high concentration phosphate solution requires several days and gives an average of 3% methemoglobin (5). Cheung *et al.* recently reported the preparation of SFHb by a DEAE-cellulose absorption technique (6). Only 11 h was required for isolation of Hb from 500 ml of RBC with an average 77% yield of Hb. However, lipid analysis was not conducted to determine the presence or absence of stroma. Lack of coagulation activity was the only criterion used to determine the absence of lipids. This criterion is not conclusive because it has been shown that disseminated intravascular coagulation (DIC) attributed to stroma contaminants in hemoglobin preparations is not necessarily due to lipid contamination. Rabiner *et al.* (7) used a 0.22-μm membrane to filter hemoglobin preparations and reported that preparations were stroma-free based on the lack of coagulant activity alone.

Rosenberry *et al.* (8) have approached the isolation of the other products from RBC, i.e., erythrocyte ghosts, by a high-volume molecular filtration technique. Their process is dependent upon passage through a series of submicron filters (100,000 Da). The process re-

[1] Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

[2] Abbreviations used: RBC, red blood cells; Hb, hemoglobin; SFHb, stroma-free Hb; DIC, disseminated intravascular coagulation; Hct, hematocrit; LFHb, lipid-free Hb; PL, phospholipid; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; SEM, scanning electron microscopy.

0003-2697/86 $3.00
Copyright © 1986 by Academic Press, Inc.
All rights of reproduction in any form reserved.

quires 2 h and is limited to very dilute suspensions of RBC [hematocrit (Hct) < 5%]. As pointed out by those authors, when protein concentration increases, flow rate decreases and leads to clogging of the filters. Although the methodology described by those authors is a reasonable approach for isolation of erythrocyte ghosts, it is not appropriate for isolation of large quantities of Hb. It is calculated that from 30 ml of washed packed cells only about 10 g Hb can be processed in several hours.

The term lipid-free Hb (LFHb) is preferred to SFHb to clearly distinguish that Hb prepared in the manner described below is free of membrane lipid. Moreover, the term SFHb has been ascribed to some Hb preparations which indeed contain contaminating membrane lipids. The principle of the process described here has been developed from our experience with dialysis encapsulation for preparation of carrier erythrocytes (9–15) and with hollow-fiber dialysis of erythrocytes (16). The most important feature of the process is based on controlled dialysis of RBC to prevent severe osmotic shock of the cells which leads to complete rupture of the RBC membrane and subsequent lipid contamination. Hb prepared by controlled dialysis and ultrafiltration is devoid of lipid and is 99% pure by electrophoretic and HPLC analysis.

MATERIALS AND METHODS

Bovine blood was collected in a 1-liter container (0.1 mg/ml, heparin) from Brahma/Hereford cattle. RBC were isolated from whole blood by centrifugation at 1000*g* and then washed three times in 5 vol of 0.9% NaCl. Washed packed cells were adjusted to 60% Hct with phosphate buffer, 15 mM K_2HPO_4, pH 7.2, 2 mM $MgCl_2$, 10 mM glucose, 134 mM NaCl. RBC were then dialyzed against 18 liters of 100 mosmol/kg (0.05 mM K_2HPO_4, 50 mM NaCl) dialysis buffer with a 1-m^2 kidney dialysis hollow-fiber cartridge (Travenol, Oak Park, Ill.) until 160–180 mosmol/kg osmotic pressure (Stage I, Fig. 1). Flow rates of the cells and dialysis buffer were 200 and 2000 ml/min, respectively. The dialyzed RBC were then processed through three 0.1-μm pore hollow-fiber units (1×10^6 mol wt cut off, Amicon Corp., Danvers, Md.). The combined surface

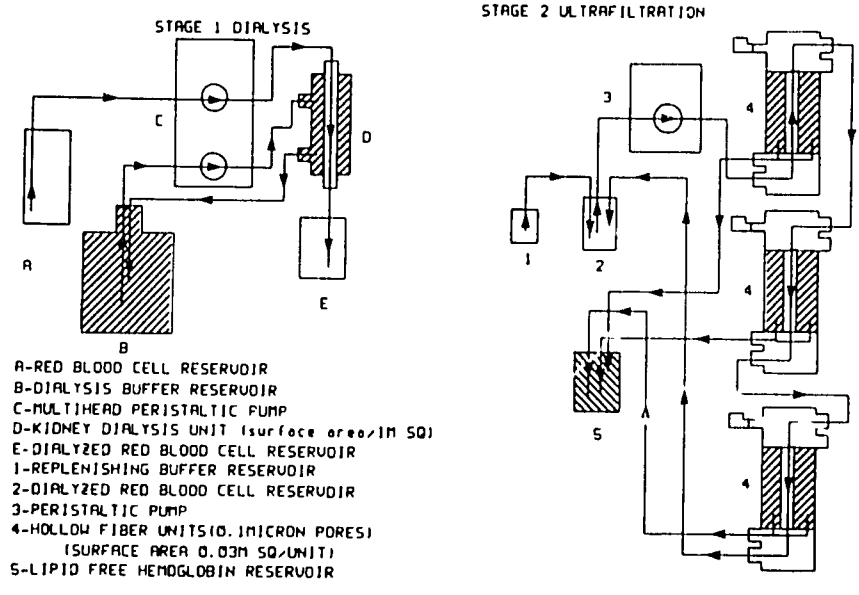

FIG. 1. Lipid-free hemoglobin apparatus.

area of the three ultrafiltration cartridges was 0.09 m^2. Dialyzed cells were maintained at a constant volume by replenishing the volume lost to ultrafiltration with a 160 mosmol/kg phosphate buffer. Lower osmotic pressure of the phosphate buffer was achieved by reduction of the NaCl content. During ultrafiltration (Stage 2, Fig. 1), the flow rate was 200 ml/min at 8–10 psi column pressure. The ultrafiltrate was then freeze-dried and stored at ambient temperature. All tubing and connectors were hemo-compatible.

Bovine Hb was also prepared by a hypotonic dilution technique. Washed packed cells were hemolyzed by diluting to 160 mosmol/kg with ice-cold distilled water. Afterward, the hemolyzed RBC solution was processed through the ultrafiltration system (Stage 2, Fig. 1) and the Hb was collected and freeze-dried. Processing through the 0.1-μm hollow fiber is similar to the preparation described by Rabiner *et al.* (7). Finally, bovine Hb was prepared from RBC (75% Hct) which were diluted fourfold in distilled water at 4°C. The hemolysate was centrifuged at 10,000*g* for 20 min. The supernatant was saved and freeze-dried. The latter preparations were made only for comparison with the Hb in the following lipid analysis.

An aliquot of the freeze-dried Hb was resuspended in 2 ml of distilled water at 10–12 g%. One-hundred-milliliter round-bottom flasks containing Hb were placed in an ice bath and 20 ml of ice-cold methanol was slowly added while stirring with a magnetic stir bar. The contents of the flask were filtered through silanized glass wool. The filtrate was collected in a 100-ml flask and immediately roto-evaporated to dryness. A small amount of benzene and ethanol was added to the flask to prevent bumping and to remove final traces of water. The dried lipid was then dissolved in 5 ml of chloroform:methanol (19:1, v/v). Extraction of the lipid from the flask was repeated three times. Each rinse solution was then filtered through a medium porosity sintered glass filter. Filtrate was reduced to dryness under argon gas. Samples were then subjected to a Folsch wash (17) and concentrated for TLC and other analysis (18).

For subsequent analysis of lipids, all solvents except diethylether and acetic acid were glass distilled and all solvents were purged with argon before use. Finally, all procedures were carried out under an argon atmosphere. TLC plates used were coated with Silica gel 60, (E Merck, Rahway, N.J.). To separate lipids into classes, a hexane:diethylether:acetic acid (60:40:2, v/v/v) solvent system was used. Lipids were visualized by iodine vapor. Phospholipids (PLs) which remained at the origin were further chromatographed by developing the plate in the same direction with a chloroform:methanol:ethanol:acetic acid:water (65:25:25:5:4, v/v/v/v/v) solvent system. Migration of lipid standards of known composition was used to identify lipid unknowns.

After the PL bands were outlined and the iodine had evaporated from the plates, each band was scraped directly into a 15-ml glass test tube. Adjacent areas of silica corresponding in size and position to the area containing PL also were scraped into test tubes. The silica gel was extracted three times with 2 ml of a mixture of methanol–chloroform (2:1, v/v), the combined extracts were evaporated to dryness and the residues were dissolved in 7 ml of a mixture of chloroform–methanol (19:1, v/v). Aliquots were then analyzed for phosphorus according to the procedures of Duck-Chong (19).

Cholesterol was analyzed automatically on a Gilford System 3500 Computer Directed analyzer. This system is designed primarily for analyzing serum cholesterol and triglycerides. However, the cholesterol end products (i.e., standards) for calibration of the instrument are prepared in isopropanol. Therefore, after reducing 30-, 60-, and 100-μl aliquots of the extracted lipids to dryness, the residues were dissolved in 200 μl of isopropanol and analyzed automatically. Reference cholesterol of known concentration in 200 μl of isopropanol was used to establish a standard curve for quantitation of the unknown.

LFHb preparations were subjected to so-

dium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE). A 4% stacking gel with a 12.5% running gel was used with 10 μl of LFHb solution on minivertical slab gel apparatus (Bio-Rad, Richmond, Calif.) at 15 mA constant current for approximately 1 h. A discontinuous Tris–glycine–SDS buffer system was used (20). After electrophoresis, the gel was then silver stained with a silver stain kit (Polysciences, Inc., Warrington, Pa.).

High-pressure liquid chromatography (HPLC) analysis was conducted on the LFHb with an instrument (LCD/Milton Roy HPLC, Riviera Beach, Fla.) equipped with a variable wavelength spectrophotometric detector. A Waters Protein-Pak DEAE 5PW column (7.5 × 75 mm) was used. Protein (10 μl injection) was eluted with a linear NaCl gradient (20 mM Tris, pH 9.2; 0–500 mM NaCl). Samples were scanned at 280 nm and protein peaks were integrated for Abs_{280}. Spectrophotometric analyses of LFHb samples were conducted from 400–600 nm.

The morphology, size, number, and Hb concentration in the RBC were monitored throughout the preparative procedure. Cell size, number, and Hb concentration were determined with a Coulter Counter equipped with a channel analyzer and a Hemoglobinometer (Coulter Electronics, Hialeah, Fla.).

Cells (0.1 ml) for scanning electron microscopy (SEM) were added to 10 ml of 1% glutaraldehyde prepared in similar osmotic pressure phosphate buffer. Cells were allowed to fix for 1 h with constant agitation at ambient temperature. After fixation, cells were washed once in buffer and twice in distilled water, and then dehydrated in 50% ethanol. Cells were then resuspended in 75% ethanol, a drop of which was transferred to a mounted coverslip. Cells were allowed to air-dry, then coated with gold.

RESULTS

The isolation scheme is divided into five steps: (i) Collection of whole blood, (ii) centrifugation of whole blood and washing of cells three times in buffer, (iii) passage of cells through kidney dialysis unit, (iv) ultrafiltration of dialyzed cells, and (v) collection and lyophilization of LFHb. Figure 1 describes the procedure and process. Dialysis of the bovine RBC to a predetermined osmotic pressure 160 mosmol/kg (9,13) allowed the RBC membrane to become permeable to Hb without rupture of the membrane. Steps 1 and 2 require about 3 h for 2 liters of whole blood. Step 3 requires only 10–15 min and Step 4 requires about 4–5 h for 500 ml of packed cells. Stage 1 dialysis (Fig. 1) requires a 1-m^2 surface area kidney dialysis cartridge. Cartridges are used once and discarded. An 18-liter dialysis buffer reservoir can be used to process 1–2 liters of packed cells. Stage 2 ultrafiltration requires a longer processing time because the protein must pass through the 0.1-μm pores in the hollow fibers. Processing time can be reduced if a larger surface area hollow-fiber cartridge is used. As dialyzed cells are recycled through the hollow fibers, volume is replenished by use of a closed vessel filled with a 160 mosmol/kg 10 mM KH_2PO_4, pH 7.2, 70 mM NaCl solution.

The data in Table 1 summarize LFHb isolation from seven different runs. In general, 400–500 ml of cells can be processed in 4–5 h. Yield of LFHb from the ultrafiltration process averages 91.1%. Actual recovery of LFHb may be less due to some loss of cells in handling and because of dead volume in both Stage 1 and Stage 2 of the apparatus. Accordingly, hematological parameters of RBC were recorded. Processed cells contained only 4% of their original Hb (Table 2). Also, processed cells were one-half of the volume of dialyzed cells.

SEM photomicrographs of normal, dialyzed, and processed cells reveal several interesting features (Fig. 2a–c). Bovine RBC appear as normal biconcave diskocytes whereas dialyzed cells appear as uniformly swollen cells. Dialyzed cells, unlike RBC ghosts, contain normal amounts of Hb and, although they are porous, the holes in the membrane are not visible. Random protrusions are observable on

TABLE 1

LIPID-FREE HEMOGLOBIN ISOLATION

Vol. of packed RBC (ml)	Starting Hb (g)	Ultrafiltration time (h)	LFHb recovered (g)	% Yield of Hb
275	26.7	2	23.5	88.3[a]
275	25.3	3	20.0	90.6
1680	166.3	14	128.0	84.2
500	133.0	4	117.0	89.0
415	95.9	5	86.4	95.5
669	153.7	5	151.3	98.4
570	107.2	5	85.1	91.8

[a] % Yield of Hb is calculated as follows:

Amt LFHb recovered ÷ (LFHb + Hb remaining in processed RBC) × 100.

% Yield does not take into account loss of cells due to dead volume in apparatus.

most of the dialyzed cells. Once Hb is extracted by ultrafiltration the cells appear mainly as empty ghosts (Fig. 2c). If RBC are hemolyzed by direct dilution, cells appear mainly as amorphous stroma (Fig. 2d). It is this form of stroma that can associate with Hb and ultimately contaminate the Hb preparation.

For comparison and for emphasis on the importance of dialysis on the nature of the isolated Hb, RBC were hemolyzed by dilution in distilled water and then processed by ultrafiltration. Significant amounts of phospholipid and cholesterol were recovered with the Hb collected by ultrafiltration (Table 3). If RBC were hemolyzed by direct lysis in distilled water followed by centrifugation at 10,000*g*, lipid was also found with the Hb contained within the supernatant (Table 3). Both of these methodologies are similar to that used by Rabiner *et al.* (7). Hb isolated by the dialysis–ultrafiltration technique is thus called LFHb.

Such LFHb migrates as a single band on SDS–PAGE (data not shown). HPLC analysis of LFHb yields one major peak at 14.4 min elution time. A minor protein peak (probably carbonic anhydrase) followed the elution of LFHb. Integration of peak areas yields a 99.6% pure Hb sample (Fig. 3). A visible spectra between 400 and 600 nm yields two broad peaks at 541 and 576 nm, characteristic of oxyhemoglobin (data not shown).

DISCUSSION

We describe a dialysis, ultrafiltration process for preparation of LFHb. The process is highly dependent on the manner in which the osmotic pressure of the cells is reduced. Reduction of the osmotic pressure of the buffer containing the RBC is achieved by controlled hollow-fiber dialysis (16). RBC swell and become permeable to Hb without appreciable cellular lysis. Thus, in the ultrafiltration step, only Hb diffuses through the 0.1-μm pores. If osmotic pressure is reduced by direct dilution in water followed by ultrafiltration, significant lipid contamination is observed. Consequently, advantage is taken of the fact that, under controlled dialysis conditions, bovine RBC do not completely lyse.

Although the term SFHb has been applied to different Hb preparations (3,7), the only ev-

TABLE 2

HEMATOLOGIC PARAMETERS OF PROCESSED CELLS

Isolation stage	Hb ($g \times 10^{-10}$/cell)	Volume (Fl)
Washed packed RBC	14.6[a]	47.1
Stage I	15.2	58.0
Stage II	0.6	27.5

[a] Data are the mean of four experiments.

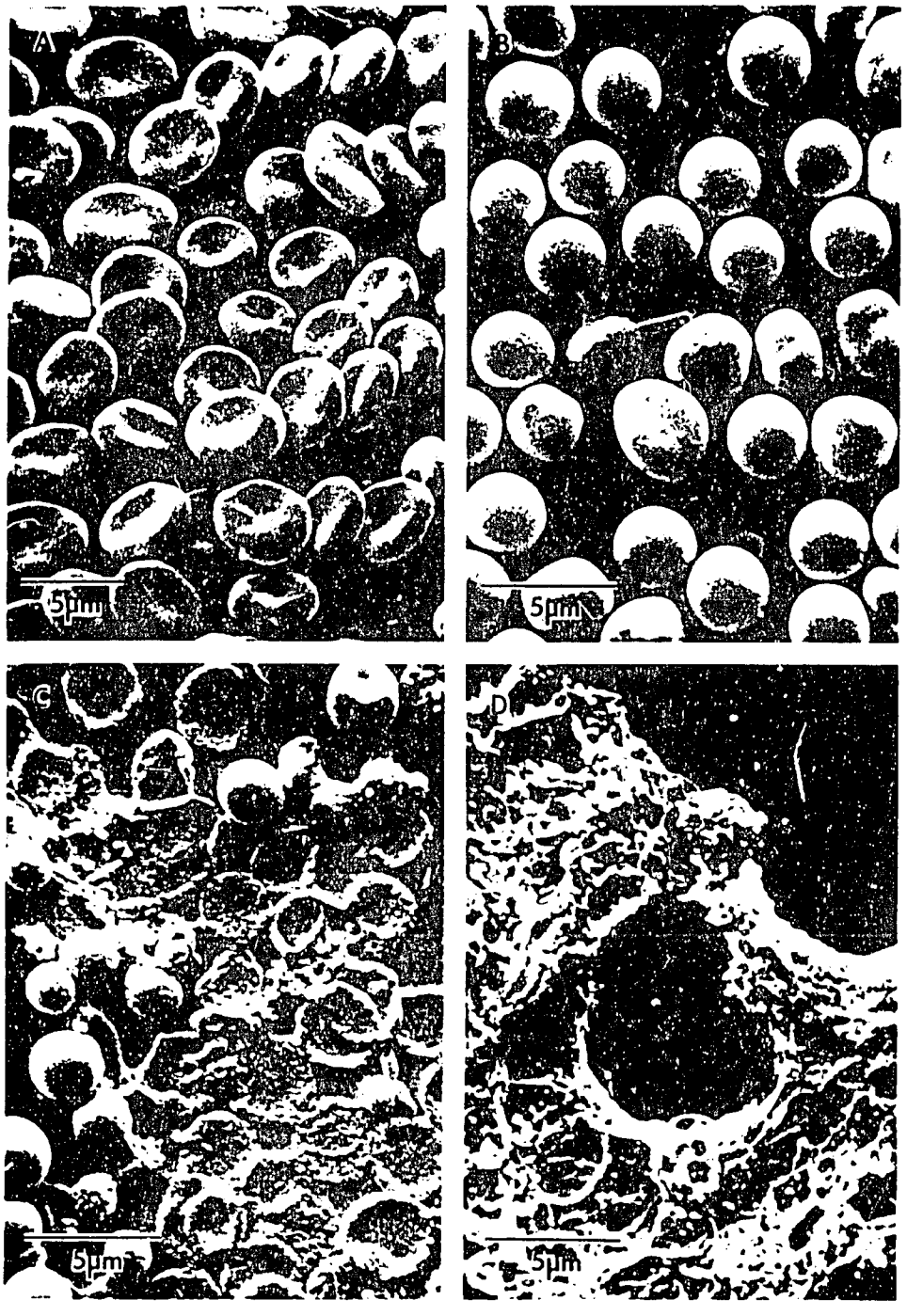

FIG. 2. Scanning electron microscopy micrographs of RBC. (a) Normal washed bovine RBC, (b) dialyzed RBC, (c) RBC ghosts, (d) stroma from RBC.

TABLE 3

LIPID CONTENT OF BOVINE HEMOGLOBIN PREPARATIONS

HB sample	Cholesterol	P_i [a]	SM	PS	PE
Cells hemolyzed in distilled water and processed by ultrafiltration	7.3[b]	0.51	14.1	1.71	3.05
Cells hemolyzed in distilled water and centrifuged	2.2	0.32	3.26	0.75	1.84
Dialyzed cells plus ultrafiltration	0.0[c]	0.0	0.0	0.0	0.0

[a] P_i = inorganic phosphorous, SM = sphingomyelin, PS = phosphatidyl serine, PE = phosphatidyl ethanolamine.
[b] Data are the mean and are expressed as mg lipid/g Hb.
[c] There were no detectable lipids from the TLC plate.

idence for this claim is the lack of enhanced clotting times for animals which previously received the Hb preparations. We present the first Hb purification in which lipid analysis shows Hb to be lipid free. De Venuto *et al.* (5) presented a crystallization method for SFHb in which no coagulant activity was found but no lipid analysis was conducted. Preliminary experiments with bovine Hb in this laboratory indicate that this procedure is not appliable to bovine Hb. Others claim that toluene precipitation may lead to irreversible binding of toluene to the Hb (1). The process described here yields a Hb preparation which is greater than 99% pure by HPLC analysis. Moreover, the LFHb migrates as a single band on SDS-PAGE. The minor protein contamination which appears on the DEAE column is probably carbonic anhydrase.

A significant advantage of this process over previous filtration methods is that hollow fibers were used instead of flat membranes. Rosenberry *et al.* (8) reported the use of filtration technique but their process required very dilute RBC solutions (5% Hct) to prevent clogging of the filters. No clogging of hollow fibers was observed with the present configuration. A considerable improvement in the time required for ultrafiltration is possible if the surface area of the ultrafiltration hollow fibers is

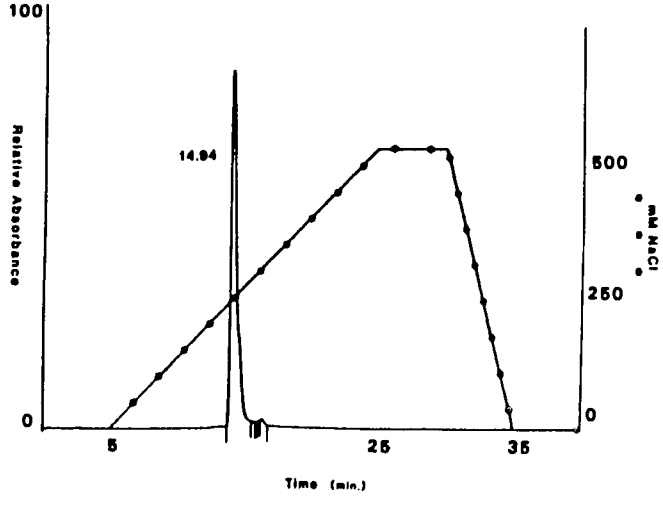

FIG. 3. Bovine hemoglobin HPLC analysis.

increased by using larger cartridges. This process is appropriate for Hb from any mammalian species in which dialysis can be used to open the membrane without destruction of the membrane. For example, dialysis has been used to prepare RBC to entrap or encapsulate a variety of exogenous agents without complete rupture of the membrane. Research from this laboratory indicates that human, bovine, ovine, caprine, equine, and murine RBC are sufficiently stable to undergo dialysis (12,21,22). However, it is likely that the reduced osmotic pressure used here for bovine (160–180 mosm) will not be appropriate for other RBC species, but rather selection of the osmotic pressure should be that used for hypotonic dialysis for entrapment of exogenous agents (12). Preliminary experiments with isolation of human LFHb verifies this hypothesis in that 110–120 mosmol/kg is optimum for isolation of LFHb from human RBC.

Hb prepared to be lipid free might have several useful applications. First, Hb used for entrapment in liposomes would be more desirable if no contaminating lipids were available for insertion into the lipid vesicle (23). LFHb has potential usefulness in the development of artificial blood diets for blood-feeding arthropods (24–26). Thus, the need for a Hb free of lipid has been established in the preparation of artificial blood for transfusion and in the preparation of artificial diets for blood-feeding arthropods.

ACKNOWLEDGEMENTS

The authors thank C. Ayala for technical assistance with the lipid analysis, Dr. A. Chen for use of his HPLC equipment, and R. Droleskey for the SEM micrographs preparation.

REFERENCES

1. Riggs, A. (1981) Methods in Enzymology (Antonini, E., Rossi-Bernardi, L., and Chiancone, E., eds.), Vol. 76, pp. 5–29, Academic Press, Orlando, Fla.
2. Schoeder, W. A., and Huisman, T. H. J. (1980) The Chromatograph of Hemoglobins, Dekker, New York.
3. Rabiner, S. F., Helbert, R. J., Lopas, H., and Friedman, L. H. (1967) *J. Exp. Med.* **126**, 1127–1142.
4. DeVenuto, F., Friedman, H. I., Neville, J. R., and Peck, C. C. (1979) *Surg. Gynecol. Obstet.* **149**, 417–437.
5. DeVenuto, F., Zuck, T. F., Zegna, A. I., and Moores, W. Y. (1977) *J. Lab. Clin. Med.* **89**, 509–516.
6. Cheung, L. C., Storm, C. B., Gabriel, B. W., and Anderson, W. A. (1984) *Anal. Biochem.* **137**, 481–484.
7. Rabiner, S. F., O'Brien, K., Peskin, G. W., and Friedman, L. H. (1970) *Ann. Surg.* **171**, 615–622.
8. Rosenberry, T. L., Chen, J. F., Lee, M. M. L., Moulton, T. A., and Onigman, P. (1981) *J. Biochem. Biophys. Methods* **4**, 39–48.
9. DeLoach, J. R., Harris, R. L., and Ihler, G. M. (1980) *Anal. Biochem.* **102**, 220–227.
10. DeLoach, J. R., Culler, K., and Harris, C. L. (1980) *J. Appl. Biochem.* **2**, 177–182.
11. DeLoach, J. R., and Barton, C. (1981) *Amer. J. Vet. Res.* **2**, 667–669.
12. DeLoach, J. R., and Sprandel, U. (eds.) (1985) Red blood cells as carriers for drugs. *Bibl. Haematol.* **51**, Karger, Basel.
13. DeLoach, J. R. *et al.*, (1982) U.S. Patent 4327710.
14. DeLoach, J. R. *et al.*, (1983) U.S. Patent 4,389,209.
15. DeLoach, J. R. *et al.*, (1985) U.S. Patent 4,517,080.
16. DeLoach, J. R. (1985) *Amer. J. Vet. Res.* **46**, 1089–1091.
17. Folch, J., Lees, M., and Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–504.
18. Spates, G. E., and DeLoach, J. R. (1986) *Comp. Biochem. Physiol.* **83A**, 667–671.
19. Duck-Chong, C. G. (1978) *Lipids* **14**, 497–499.
20. Leammli, U. K. (1970) *Nature (London)* **680**, 227–228.
21. DeLoach, J. R., and Droleskey, R. (1986) *Comp. Biochem. Physical* **83A**, 772–777.
22. DeLoach, J. R., and Droleskey, R. (1986) *Amer. J. Vet. Res.*, in press.
23. Hunt, C. A., Burnette, R. R., MacGregor, R. D., *et al.* (1985) *Science* **1165**, 1980–1982.
24. DeLoach, J. R., and Taher, M. (1982) *Bull Ent. Res* **663**, 72–74.
25. DeLoach, J. R., and Spates, G. (1984) *Southwest. Entomol.* **9**, 28–32.
26. Kabayo, J., Taher, M., and Vanderfloedt, A. (1985) *Bull. Ent. Res.* **75**, 635–640.

Comp. Biochem. Physiol. Vol. 79A, No. 3, pp. 383–386, 1984
Printed in Great Britain

0300-9629/84 $3.00 + 0.00
Pergamon Press Ltd

2.H-04

AN IMPROVED FREEZE-DRIED BLOOD DIET FOR MEMBRANE FEEDING OF *GLOSSINA PALPALIS PALPALIS**

J. R. DeLoach† and M. Taher‡

†U.S. Livestock Insects Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Kerrville, TX 78028, USA and ‡International Atomic Energy Agency, Vienna, Austria

(*Received* 14 *February* 1984)

Abstract—1. The effect of fetal calf serum as an additive for reconstituted freeze-dried bovine and porcine blood for feeding *Glossina palpalis palpalis* was determined.

2. Reproductive performance of tsetse flies fed reconstituted freeze-dried bovine and porcine blood supplemented with fetal calf serum was higher than that of flies fed only freeze-dried blood.

3. Sonicated freeze-dried porcine blood supplemented with fetal calf serum resulted in a reproductive performance equivalent to that of flies fed on non-sonicated freeze-dried porcine blood and fetal calf serum.

4. A 50:50 mixture of freeze-dried porcine and freeze-dried bovine blood reconstituted and sonicated resulted in a reproductive performance equivalent to the highest ever attained for *in vitro*-reared *G. p. palpalis*.

INTRODUCTION

Efforts have been made to find a suitable diet for membrane feeding of *Glossina* spp. for use in a mass rearing facility. Research by Wetzel (1980) led to the adoption of freeze-dried bovine blood as a replacement for fresh blood. In a mass rearing facility, the logistics of obtaining sterile, high-quality fresh abattoir blood is difficult. The problem of blood storage and blood quality for feeding tsetse flies has been somewhat ameliorated by the use of freeze-dried bovine blood. However, there are still some problems associated with freeze-dried blood because freeze-dried bovine blood alone is not equivalent to fresh bovine blood as a diet for tsetse flies (DeLoach and Taher, 1983). More research is needed to improve the physical and nutritional character of the reconstituted blood. The ultimate goal of *in vitro* rearing is to provide sufficient quantities of sterile male flies for a sterile insect release program.

Research in our laboratories has been directed to improving the quality of the *in vitro* diet so that it equals that of fresh animal blood. For example, the reproductive performance of *Glossina palpalis palpalis* fed *in vitro* on fresh guinea pig blood through a membrane is equal to that of flies fed directly on guinea pigs (DeLoach and Taher, 1982). The maximum obtainable PPF (pupae per mature female) was 0.098 with both feeding regimens. With freeze-dried porcine or bovine blood, a typical PPF is in the 0.075–0.080 range, which is somewhat less than the theoretical 0.105 PPF. The reproductive capacity of tsetse flies fed on freeze-dried bloods can to some extent be improved if the reconstituted blood is sonicated before use (DeLoach, 1983).

Many kinds of additives have been tested with freeze-dried blood to restore its nutritional quality to that of the original blood. For example, bovine serum albumin (BSA) added to freeze-dried bovine blood will in certain instances improve the nutritional value of the blood. As prepared, each lot of freeze-dried blood has its own intrinsic nutritional quality, with some lots better than others. When BSA is used as a supplement to a particular lot of freeze-dried blood whose nutritional value is poor, the reproductive performance of the flies will improve (DeLoach and Taher, 1983). Wetzel (1980) reported a similar effect in earlier tests.

The present test were conducted to determine whether a combination of additives and sonication with freeze-dried blood could result in a product equal to fresh bovine blood. Tsetse flies were fed on the different diets for 35 days, and survival, fecundity, and weight of offspring were observed.

MATERIALS AND METHODS

G. p. palpalis was obtained from a membrane-fed colony maintained at IAEA Laboratory (Seibersdorf, Austria). The flies were reared at 24.5 ± 0.5°C and 85% relative humidity (van der Vloedt, 1975, 1982). Fifteen newly emerged females were placed in a cage, and after 3 days, 5–7 males were introduced for breeding. Each day (except Sunday) the cages were examined for dead flies and the number of puparia produced. Puparia were individually weighed.

Control flies (4 groups of 15 flies) were fed on fresh bovine blood 6 days a week. Experimental flies (4 groups of 15 flies) were fed with the prepared diet on a 6-day regimen. Quantities of freeze-dried bovine (FDBB) and porcine blood (FDPB) were obtained from random lots stored at the IAEA. Fresh bovine blood was collected in heparin from locally obtained cattle, and fresh bovine plasma was obtained from the fresh whole blood. The experiments were conducted at the same time in order to minimize variations due to rearing and handling procedures and due to quality of flies obtained from the *in vitro* colony.

FDBB was fed alone or supplemented with 10% fetal calf serum (FCS). FDPB was fed alone, with 10% FCS, with

*Mention of a commercial product does not constitute an endorsement by USDA.

Rec'd in SCI APR 10 1990

cc BOSTIO
CDIE.

Table 1. Reproductive performance of *Glossina palpalis palpalis* fed on supplemented freeze-dried bovine blood

Diet	% survival day 18–35*	Total pupae by day 35	Pupa initial female	Pupa mature female day	Female day relative to theoretical max	Mean pupal weights (mg) ± SD 1st reproductive cycle	2nd reproductive cycle
Fresh bovine blood	94*a*†	86	1.43	0.085*a*	0.080	30.5 ± 4.4*a*	31.3 ± 3.9*a*
+ FCS	95*a*	85	1.42	0.083*a*	0.079	30.6 ± 3.7*a*	32.5 ± 3.1*a*
FDBB	86*b*	80	1.33	0.087*a*	0.074	26.8 ± 3.6*b*	29.7 ± 3.6*a*
FDBB + FCS	91*a*	89	1.48	0.091*b*	0.082	28.9 ± 3.0*a*	29.7 ± 3.6*a*

*Survival percentage is calculated as follows:

$$\frac{\text{Actual mature female days}}{\text{theoretical mature female days or } 15 \times 18 \text{ days}} \times 100$$

†Data in columns followed by a common letter are not significantly different ($P = 0.05$); Duncan's multiple range test.

10% FCS and sonication, with 50% FDBB, and with 50% FDBB plus sonication. FCS was purchased from Serva Heidelberg, F.R.G. Sonication was as previously described (DeLoach, 1983). All blood diets were stored at −20°C until use. ATP (10^{-3} M) was added immediately before feeding. All feeding was conducted through an artificial membrane (Wetzel, 1979a and b).

RESULTS

The reproductive performance of tsetse flies fed fresh bovine blood was higher than expected. The data are included here only for comparison since the reproductive performance of tsetse flies fed bovine blood is well documented (Wetzel, 1979b, 1980). Flies fed fresh bovine blood supplemented with FCS had a reproductive performance equivalent to that of flies fed on only fresh blood. FCS did not improve the nutritional quality of fresh bovine blood. However, the reproductive capacity (PPF) of flies fed on FDBB was nearly equal to that of flies fed on fresh blood. The highest reproductive capacity of flies was exhibited when flies were fed FDBB plus FCS (Table 1). Offspring weights of the first reproductive cycle were significantly lower for flies fed FDBB as compared with those for flies fed any of the other test diets. There was no difference in the mean pupal weights from the second reproductive cycle (Table 1). Survival of mature females (day 18–35) was slightly less for flies fed on FDBB.

Fresh pig blood from the abattoir was freeze-dried, and a direct comparison between fresh and freeze-dried blood was made. There was no difference in the reproductive performance of flies fed on the two diets (Table 2). The overall reproductive performance of flies fed on FDPB was markedly enhanced by the addition of FCS combined with sonication. This level of reproduction is equivalent to that obtained for flies fed *in vivo* on guinea-pigs (DeLoach and Taher, 1983). A somewhat surprising result was obtained when FDPB and FDBB were fed in combination. Reproductive capacity of flies was equivalent to that of flies fed on FDPB. More significantly, however, sonication of the FDPB:FDBB diet resulted in a product whose nutritional value was excellent for *G. p. palpalis*. A 0.100 PPF approaches the theoretical maximum, 0.105 PPF, and is equivalent to the highest reproductive capacity attained by *in vitro*-reared *G. p. palpalis* (DeLoach and Taher, 1982).

DISCUSSION

A problem inherent to mass rearing of tsetse flies is their relatively low fecundity as compared with that of other Diptera. *G. p. palpalis* produce only one offspring every 9–9.5 days after reaching sexual maturity. The theoretical 0.105 PPF is based on a 9.5-day reproductive cycle (van der Vloedt, 1975, 1982). Another dipteran, *Stomoxys calcitrans* (L.), produces 40 eggs per day after reaching sexual maturity (Spates, 1979). Thus, it is of great significance to maximize the reproductive capacity of membrane-fed *G. p. palpalis*. It has been demonstrated that flies fed through a silicone membrane on a nutritionally

Table 2. Reproductive performance of *Glossina palpalis palpalis* fed on supplemented freeze-dried porcine blood

Diet	% survival day 18–35*	Total pupae by day 35	Pupa initial female	Pupa mature female day	Pupa mature female day relative to theoretical max	Mean pupal weights (mg) ± SD 1st reproductive cycle	2nd reproductive cycle
Fresh porcine blood	98*a*	89	1.48	0.084*a*	0.082	30.7 ± 3.5*a*	30.1 ± 3.7*a*
FDPB	95*a*	87	1.45	0.085*a*	0.081	29.8 ± 5.5*a*	31.0 ± 5.0*a*
FDPB + FCS	88*b*	93	1.55	0.098*b*	0.086	32.2 ± 3.6*b*	31.2 ± 4.0*a*
FDPB + FCS sonicated	96*a*	99	1.74	0.099*b*	0.096	31.7 ± 4.1*ab*	31.0 ± 4.6*a*
FDPB + bovine serum	94*a*	88	1.47	0.086*a*	0.082	29.6 ± 3.2*ab*	30.2 ± 3.8*a*
FDPB:FDBB 50:50	92*ab*	8[illegible]	1.40	0.084*a*	0.078	30.2 ± 3.7*a*	29.1 ± 4.4*a*
FDPB:FDBR 50:50 sonicated	98*a*	107	1.78	0.100*b*	0.099	32.2 ± 4.0*b*	32.0 ± 3.8*a*

*Survival percentage is calculated as follows:

$$\frac{\text{Actual mature female days}}{\text{theoretical mature days or } 15 \times 18 \text{ days}} \times 100$$

†Data in columns followed by a common letter are not significantly different ($P = 0.05$); Duncan's multiple-range test.

adequate diet will achieve a reproductive performance equivalent to that of flies fed *in vivo* on guinea pigs (DeLoach and Taher, 1982). Consequently, the reproductive performance of *G. p. palpalis* is not limited by membrane feeding. A 0.075 PPF for a colony of 10,000 producing females results in only 750 offspring per day. Thus, if an increase to 0.1 PPF could be achieved, the production of 1000 pupae per day would be a significant increase in colony performance.

Our recent research has sought to find additives for freeze-dried bloods which will restore their nutritional value to that of fresh blood. Freeze-drying is known to increase the blood alkalinity to about pH 8.0–8.2. Moreover, freeze-drying alters levels of free amino acid in blood (DeLoach and Holman, 1983). The physical character of the freeze-dried blood is significantly altered from intact cells plus plasma to an amorphous particulate product with some large aggregate particles. Sonication of reconstituted freeze-dried blood reduces particle size to a uniform 5–10-fl particle. The reproductive performance of flies fed on sonicated reconstituted blood is significantly higher (DeLoach, 1983). Thus, the freeze-drying process is known to alter physical and chemical properties of the blood.

Several kinds of additives such as lipids, BSA, hemoglobin, and amino acids have been added to both fresh and freeze-dried bovine blood to increase its nutritional value (Moloo and Pimley, 1978; Langley *et al.*, 1978; DeLoach and Taher, 1983). Despite these attempts, very little success has been noted. Our philosophy toward selection of additives for freeze-dried blood is based on restoring the 'fresh' quality of the blood product to that of fresh whole blood. A more quantitative view is simply to find additives which replace as yet unknown factors in blood that are altered by freeze-drying. One useful approach in the present experiments has been the addition of FCS to FDBB and FDPB. Researchers have long sought to define the superior value of FCS in tissue culture media with little success (Eagle, 1955; Eagle, 1960). In both instances, 10% FCS was used on the basis of results of preliminary feeding tests with FDBB and FDPB. Fresh bovine blood supplemented with FCS expectedly was not different from fresh blood alone. FCS as a supplement to FDBB resulted in an excellent dietary blood product. *G. p. palpalis* fed on such a diet had high fecundity and normal pupal weights equivalent to those of pupae from flies fed on fresh blood.

The reproductive performance of flies fed on fresh porcine blood and FDPD was determined as a point of reference, then 10% FCS was added to FDPB. The fecundity of flies fed on this diet was superior, yielding a 0.098 PPF and 31–32 mg offspring. Sonication of the FDPB plus FCS did not improve its quality as indicated by the reproductive performance of the flies (Table 2). Although sonication or FCS supplement alone improve the quality of FDPB, they are not additive. Since sonication alters the physical character and improves the solubility of reconstituted freeze-dried blood (DeLoach, 1983), perhaps FCS has a similar effect. However, when fresh bovine serum was added to FDPB, no improvement in fecundity of flies was noted. Thus, it appears that FCS as a supplement enhances the nutritional value of FDPB biochemically rather than physically.

In the search to maximize the nutritional value of freeze-dried blood, a combined FDPB:FDBB product was tested. The fecundity was quite good at 0.084 PPF with 29–30 mg pupae. More importantly, this product was improved by its sonication. The performance of flies reached a maximum of 0.099 PPF relative to 0.105 PPF theoretical maximum with 32 mg pupae. The performance of flies in these tests fed the 50:50 mixture is somewhat higher than that for the colony flies at IAEA (van der Vloedt, personal communication)

There are now at least three procedures which improve the reproductive capacity of tsetse flies fed on reconstituted freeze-dried blood. Sonication, FCS supplement, and sonicated 50:50 blood mixture of FDPB and FDBB all yield a diet which approaches a 0.099 PPF production relative to theoretical maximum with 30–32 mg pupae. Incorporation of any one of these procedures in diet preparation should improve the reproductive capacity of a *G. p. palpalis* tsetse colony.

Acknowledgement This research was conducted in cooperation with the International Atomic Energy Agency (Research Agreement 3108 CF) Vienna, Austria.

REFERENCES

DeLoach J. R. (1983) Sonication of reconstituted freeze-dried bovine and porcine blood: effect on fecundity and pupal weights of the tsetse fly. *Comp. Biochem. Physiol.* **76A**, 47–49.

DeLoach J. R. and Holman G. M. (1983) An amino acid analysis of freeze-dried bovine and porcine blood used for *in vitro* rearing of *Glossina palpalis palpalis* (Rob-Desv). *Comp. Biochem. Physiol.* **75A**, 499–502.

DeLoach J. R. and Taher M. (1982) *In vitro* rearing of *Glossina palpalis palpalis* (Robineau-Desvoidy) (Diptera:Glossinidae) on guinea pig blood. *Bull Ent. Res.* **72**, 663–667.

DeLoach J. R. and Taher M. (1983) Investigations on development of an artificial diet for *in vitro* rearing of *Glossina palpalis palpalis* (Diptera:Glossindae). *J. econ. Entomol.* **76**, 1112–1117.

Eagle H. (1955) Nutrition needs of mammalian cells in tissue culture. *Science* **122**, 501–504.

Eagle H. (1960) The sustained growth of human and animal cells in a protein-free environment. *Proc. Natn. Acad. Sci. U.S.A.* **46**, 427–432.

Langley P. A., Pimley R. W., Mews A. R. and Flood M. E. T. (1978) Effect of diet composition on feeding digestion and reproduction in *Glossina morsitans*. *J. Insect Physiol.* **24**, 233–238.

Moloo S. K. and Pimley R. W. (1978) Nutritional studies of *in vitro* feeding techniques for *Glossina morsitans*. *J. Insect Physiol.* **24**, 491–497.

Spates G. E. (1979) Fecundity of the stable fly: effect of soybean trypsin inhibitor and phospholipase inhibitor on the fecundity. *Ann. Entomol. Soc. Am.* **72**, 845–849.

van der Vloedt A. (1975) L'elevage au laboratoire de *Glossina palpalis* et de *Glossina fuscipes quanzensis*. *Off. Intern Epizoot. Rev. Elev. Med. Vet. Pays. Trop. Suppl.* **1**, 51–62.

van der Vloedt A. (1982) Recent advances in tsetse mass rearing with particular reference to *Glossina palpalis palpalis* (Rob-Desv.) fed *in vivo* on guinea pigs. *IAEA-SM*-255/11.

Wetzel H. (1979a) Zuchtung von tsetse fleigen mit membrane fetterung. Gesellschaft fur Strahlen und Umwelt-

forschung. Munchen GSF Bericht BT171 PhD dissertatin.

Wetzel H. (1979) Artificial membrane for *in vitro* feeding of piercing suckling arthropods. *Entomol. Exp. Appl.* **25**, 117–119.

Wetzel H. (1980) The use of freeze-dried blood in the membrane feeding of tsetse flies (*Glossina p. palpalis*, Diptera: Glossinidae). *Tropenmed. Parasitology* **3**, 259–274.

Comp. Biochem. Physiol. Vol. 83A, No. 4, pp. 667–671, 1986
Printed in Great Britain

0300-9629/86 $3.00 + 0.00
Pergamon Press Ltd

POSSIBLE CORRELATIONS BETWEEN REPRODUCTIVE PERFORMANCE OF *STOMOXYS CALCITRANS* AND LIPID CONTENT OF BLOOD DIETS*

2. H- 04

G. E. SPATES and J. R. DELOACH

Veterinary Toxicology and Entomology Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, P.O. Drawer GE, College Station, TX 77841, USA. Telephone: (409) 260-9404

(*Received* 25 *July* 1985)

Abstract—1. Adult stable flies were fed blood meal diets of freeze-dried bovine or porcine blood, or fresh bovine or porcine blood, for ten consecutive generations; the reproductive performance of adults fed freeze-dried or fresh porcine blood was significantly higher ($P \leq 0.05$) than that of adults fed freeze-dried or fresh bovine blood.

2. Quantitative analyses of cholesterol, triglycerides, phospholipid phosphorus, phospholipid classes, and fatty acid composition of the diet were made.

3. There was no direct correlation between the percentage distribution of lipid classes and the fecundity of flies. However, the locations of phosphatidyl choline and sphingomyelin within the blood components may be a factor in the diet.

cc BOS TID CDIE

INTRODUCTION

Adult stable flies (*Stomoxys calcitrans*) are obligatory blood feeders for functional reproductive capacity (Hoffman, 1968; Spates, 1979). Since adult stable flies are similar to tsetse flies in their nutritional requirements (DeLoach and Spates, 1984), we have been using stable flies to test for development of an artificial blood diet. Tsetse flies (*Glossina palpalis palpalis*) have been fed *in vitro* a number of different blood products and blood derivatives (Bauer and Aigner, 1978; Langley *et al.*, 1978; Moloo and Pimley, 1978; Wetzel, 1978; Wetzel, 1980; DeLoach and Taher, 1983). Their basic requirements are that the blood be sterile and nutritionally fresh. In contrast to tsetse flies, stable flies can feed on nonsterile blood. But the source of the blood can be equally important for both species. For example tsetse flies exhibit a higher reproductive capacity when fed porcine blood than when fed bovine blood, whether the blood is fresh (Langley *et al.*, 1978; DeLoach and Taher, 1983) or freeze-dried (DeLoach and Taher, 1983). More recent feeding tests with stable flies fed freeze-dried bovine and porcine blood indicate that porcine blood is also nutritionally superior for this species (Spates and DeLoach, 1985).

Much research has been devoted to understanding the nutritional superiority of porcine blood over bovine blood (Langley *et al.*, 1978; Moloo and Pimley, 1978; DeLoach and Taher, 1983). An amino acid analysis of freeze-dried bovine and porcine blood used for *in vitro* rearing of *G. p. palpalis* revealed significant differences in the two types. Bovine blood had higher levels of lysine, methionine, threonine and phenylalanine (DeLoach and Holman, 1983). Porcine blood was higher only in isoleucine. Addition of isoleucine to bovine blood did not improve reproductive capacity of tsetse flies fed on that diet (Moloo and Pimley, 1978). Experiments in which nutrients are added to bovine blood have met with little success. Addition of lecithin to bovine blood had no effect on fly fecundity. When an amino acid mixture, a vitamin mixture, lecithin or hemoglobin were added to defibrinated bovine blood, the fecundity of tsetse flies (*Glossina morsitans*) did not increase (Moloo and Pimley, 1978). Additions of lysine adversely affected fecundity, as did the addition of linolenic acid (C18:3). The addition of bovine serum albumin to bovine blood elicited some improvement in the fecundity of *G. morsitans* (Moloo and Pimley, 1978) and of *Glossina palpalis palpalis* (DeLoach and Taher, 1983). However, the addition of lipids such as cholesterol to bovine blood caused a marked reduction in fecundity of *G. p. palpalis* (DeLoach and Taher, 1983). Thus, the cause of the nutritional superiority of porcine blood is still undetermined.

To further examine these nutritional differences between porcine and bovine blood, we have fed stable flies on freeze-dried bovine and porcine blood for ten generations, and have examined the lipids found in each blood. This analysis of lipids includes cholesterol, triglyceride, phospholipid phosphorus, phospholipid classes and fatty acid content.

MATERIALS AND METHODS

Stable flies

Stable flies used in this study were from a colony that has been maintained at our laboratory since 1969. Conditions for colony maintenance and egg collection have been described (Ivie and Wright, 1978; Bridges and Spates, 1979). The performance of stable flies fed freeze-dried or fresh blood was determined in three groups of adults (6 × 12.5) for each of ten generations. Detailed conditions for feeding,

*Mention of a commercial or proprietory product in this paper does not constitute an endorsement of this product by the U.S. Department of Agriculture.

CBP 83A—E Rec'd in SCI 1990

handling and egg collection were as previously described (Spates and DeLoach, 1985).

Blood diets

Flies were fed fresh bovine blood (FBB), fresh porcine blood (FPB), and freeze-dried bovine (FDBB) and porcine (FDPB) blood prepared in Vienna, Austria and College Station, TX, denoted as V and CS, respectively. Freeze-dried bovine (FDBB-V) and porcine (FDPB-V) bloods were prepared at the International Atomic Energy Laboratory, Seibersdorf, Austria, with a commercial apparatus (Wetzel, 1980). Freeze-dried bovine (FDBB-CS) and porcine (FDPB-CS) bloods were prepared on a laboratory freeze drier in College Station, Texas. Freeze-dried bloods were prepared in 1980 and reconstituted over a 10-month period in 1983. Enough blood was reconstituted (4.1 ml deionized water/g blood) to feed each generation of adults and for lipid analysis. Reconstituted blood was stored at −20°C until used. Porcine (FPB) and bovine (FBB) blood was collected weekly in heparinized vacutainers and held at 4°C for feeding adults or stored at −20°C for lipid analysis. Prior to feeding, blood was warmed to 30°C. Flies were allowed to feed to repletion from dental rolls saturated with blood.

Chemicals

All solvents except acetic acid and diethyl ether were redistilled before use. Solvents also were saturated with argon before they were used. TLC plates (20 × 20 cm) precoated with silica gel H (250 μm thick) were obtained from Analtech, Newark, DW. Triglycerides, cholesterol, cholesterolesters and borontrifluoride in methanol were obtained from Sigma Chemical Co., St Louis, MO. All other TLC and GLC standards were obtained from Supelco, Inc., Bellefonte, PA. Reagent kits for measuring cholesterol and triglycerides were obtained from Worthington Diagnostics Co., San Francisco, CA, and Dow Diagnostics, Indianapolis, IN, respectively.

Lipid extraction

Lipid extractions were carried out on reconstituted FDBB-V, FDBB-CS, FDPB-V, FDPB-CS, FBB and FPB. Blood samples from various dates were thawed and pooled for lipid extractions. Three 5-ml aliquots were taken from each of the pooled samples, slowly added to 50 ml of ice-cold methanol and slowly stirred for 5 min with a magnetic stirring bar. Afterwards, 100 ml of ice-cold chloroform were added and the mixtures were allowed to stir for an additional 20 min. The mixtures were allowed to stand for 0.5–1.0 hr to allow the denatured hemoglobin to settle, and then the solvents were filtered through two layers of prewashed Whatman No. 1 filter paper into 300-ml round-bottom distilling flasks. The beakers used during extraction were rinsed three times with 25 ml of chloroform–methanol (2:1, v/v). The combined filtrates were reduced to near dryness on a Buchi™ rotovaporator at 30°C. An argon trap was placed between the vacuum bleed of the rotovaporator and the cylinder of argon to ensure that the lipid extracts were exposed to a minimum of oxygen. Thereafter, the flasks were rinsed three times with 20 ml of freshly prepared chloroform–methanol (19:1, v/v); rinses were filtered through a sintered glass funnel (medium porosity) having a 1.0 cm layer of prewashed sodium sulfate. Combined rinses were reduced to dryness on the rotovaporator. Freshly made chloroform–methanol (2:1, v/v) was used to transfer the lipid extracts to conical, graduated centrifuge tubes, and the nonlipid materials were removed according to the method of Folch *et al.* (1957).

Chromatographic procedures

Prior to use, TLC plates were cleaned in a chromatographic tank containing 200 ml of a mixture of chloroform–methanol–ethanol–acetic acid–water (65:25:25:5:4, v/v). The solvent was allowed to ascend to the top of the plates. Plates were air-dried and then activated in an oven at 110°C for 30 min. An aliquot (500 μl) of the washed lipid extracts was reduced to 50 μl with a stream of argon, then streaked onto the TLC plates with small-bore capillary tubing. Samples and standards were placed on a line 2.5 cm from the bottoms of the plates, and eluting solvents were allowed to ascend to 2.5 cm from the tops of the plates. Lipid extracts were separated into major classes of lipids by TLC using a solvent mixture of hexane–diethyl ether–acetic acid (30:20:1, v/v).

The TLC zone containing phospholipids was visualized with iodine vapor, scraped from the plate, and placed into a 15 ml glass culture tube. Silica gel was extracted seven times with 5 ml of a mixture of methanol–chloroform (1:1, v/v); each extraction involved vigorously vortexing the tube containing silica gel and solvent, followed by low speed centrifugation. Phospholipid extracts were reduced with nitrogen to a volume of 50 μl or less, and then subjected to TLC again for separation of individual phospholipids.

Classes of lipids and individual phospholipids were identified by spotting reference lipids onto TLC plates and by exposing the plates to iodine vapor. Phosphatidyl ethanolamine and phosphatidyl serine also were detected with ninhydrin spraying.

Analysis of phospholipid phosphorus

After the PL bands were outlined and the iodine had evaporated from the plates, each band was scraped directly into a 15-ml glass test tube. Adjacent areas of silica corresponding in size and position to the area containing PL also were scraped into test tubes. The silica gel was extracted three times with 2 ml of a mixture of methanol–chloroform (2:1, v/v), and then the combined extracts were evaporated to dryness and the residues dissolved in 7 ml of a mixture of chloroform–methanol (19:1, v/v). Aliquots were then analyzed for phosphorus according to the procedures of Duck-Chong (1978).

The extracted silica gel from sample and reference PL, as well as corresponding silica gel blanks, were also analyzed for phosphorus using a modification of the Duck-Chong (1978) method. A 100-μl aliquot of magnesium nitrate in methanol (10%, w/v) was pipetted onto the silica gel and the methanol evaporated to dryness in a heated water bath (85–100°C). Then the tubes were heated for 35 sec by holding them just above the blue flame of a Bunsen burner. After cooling, 2 or 3 ml of 1 M HCl was added to each tube and the tubes were vortexed vigorously, capped with a cleaned marble, then heated in a water bath (90–95°C) for 25 min. When tubes cooled they were vortexed and centrifuged. A 1-ml portion of the acidic extract was then transferred to another tube and the routine phosphorus assay was carried out. When less than 1 ml of acidic extract was used, the volume was adjusted to 1 ml with additional 1 M HCl.

Transesterification of saponifiable lipids

The lipid samples (100 μl) were placed in glass mini-aktor tubes (Applied Science, Deerfield, IL) and reduced to dryness with a stream of argon. Borontrifluoride in methanol (1 ml) was added to each tube, and then the tubes were capped and placed in a 100°C water bath for 30 min. After brief cooling, 2 ml of distilled water was added to each tube. The fatty acid methylesters (FAME) were extracted from the methanol and water with petroleum ether (three times with 5 ml). Combined petroleum ether extracts were washed with 3 ml of distilled water; then a small amount of anhydrous sodium sulfate was used to remove final traces of water from the petroleum ether. The petroleum ether extract was reduced to dryness and the residue was dissolved in 30 μl of petroleum ether for GLC analysis of the FAME.

FAME samples were analyzed on a Tracor MT 220 GLC equipped with a FID detector and a 1.83 m × 3.0 cm i.d.

U-shaped glass column packed with 10% (w/w) SP-2330 on 100/120 mesh Supelcoport (Supelco, Inc.). Operating conditions were: column temperature program, 125–200 C–2 C/min; inlet and detector temperature, 250 C; H_2 and air flow rates to the detector, 40 and 120 cm^3/min, respectively; and N_2 flow rate, 30 cm^3/min.

Identification and quantitation of FAME in samples were accomplished through the use of reference samples of FAME and a Hewlett-Packard model 3390A reporting integrator.

Cholesterol and triglyceride analysis

Cholesterol and triglycerides were analyzed automatically on a Gilford System 3500 Computer Directed Analyzer. This system is designed primarily for analyzing serum cholesterol and triglycerides. However, the cholesterol and triglyceride end products (i.e. standards) for calibration of the instrument are in isopropanol. Therefore, after reducing 30-, 60- and 100-μl aliquots of the extracted lipids to dryness, the residues were dissolved in 200 μl of isopropanol and analyzed automatically. Reference cholesterol and triglycerides of known concentration in 200 μl of isopropanol were used to establish a standard curve for quantitation of the unknown.

RESULTS

The mean fecundity and percent egg hatch for the parental and ten subsequent generations of stable flies are summarized in Table 1. Flies fed FDBB-CS and FDBB-V exhibited significantly higher fecundity ($P = 0.05$) than did flies fed fresh bovine blood. Among the flies fed the three porcine blood diets, there was no significant difference ($P = 0.05$) in reproductive performance. The mean fecundity of flies fed on the FPB, FDPB-V and FDPB-CS was significantly greater than the fecundity of flies fed on any of the bovine blood diets. There was no difference in the percent hatch of eggs from flies fed on any of the diets except FBB, which was significantly lower at 74.2%.

The cholesterol content of the FPB and FBB was significantly greater than that of the FDPB-V and FDPB-CS (Table 2). With a cholesterol content of 91.2–129.8 mg/dl, all the diets were within normal values for bovine and porcine blood. No correlation between cholesterol content of the diet and fecundity of the flies can be made.

Analyses of the triglyceride content of the various blood diets (Table 3) revealed several significant differences ($P = 0.050$). The concentration of triglycerides in FDPB-CS was at least two-fold higher than in any of the other diets. Even though FDPB-V (36 mg/dl), FPB (74.2 mg/dl) and FDPB-CS (174.5 mg/dl) exhibited to two- to four-fold difference in concentration, the fecundity of flies was not different (Table 1). It should be noted that the freeze-dried bloods prepared in College Station were not derived from the fresh bloods used in these experiments; however, they were prepared from blood obtained from similar breeds of animals.

Although phospholipid phosphorus analyses revealed differences among the blood diets (Table 4), there was no correlation between phospholipid phosphorus content of the diet and the fecundity of the flies (Table 1).

The phospholipids from the various diets were separated into the following classes: sphingomyelin (SM), phosphatidyl choline (PC), phosphatidyl inositol (PI), phosphatidyl ethanolamine (PE) and phosphoglyceride (PG). The percentages of total phospholipids in each class are shown in Table 5. The concentrations of SM in the three bovine blood diets were not significantly different ($P = 0.050$), but they were significantly different in the three porcine diets. All but FDPB-V contained less SM than was found in the three bovine bloods. Although there were marked differences in the PC content of the blood diets, no correlation with fly fecundity could be made. An interesting finding was the extremely high PE content of the FPB; possibly there was a loss of PE from FDPB-V and FDPB-CS due to decomposition, since PE is quite labile. Also, since lyso PE migrated the same as SM, it was included in the percent SM.

Table 1. Reproductive performance of parental and ten generations of stable flies fed on fresh or freeze-dried bovine and porcine blood

Diet	Fecundity egg/♀/day	Egg hatch (%)
FBB	27.1a*	74.2a
FDBB-V	36.8b	87.1b
FDBB-CS	35.4b	91.2b
FPB	49.4c	87.8b
FDPB-V	48.3c	91.0b
FDPB-CS	50.0c	93.6b

*Data are means of eleven generations of flies. Data in same column followed by a common letter are not significantly different ($P = 0.05$).

Table 2. Cholesterol content of blood diets

Diet	$\bar{X} \pm$ SEM ($N = 9$) (mg/dl)
FDPB-CS	91.2 ± 3.8a*
FDPB-V	104.6 ± 3.4a,b
FDBB-V	108.2 ± 5.2a,b,c
FDBB-CS	117.4 ± 3.8b,c,d
FBB	125.7 ± 4.6c,d
FPB	129.8 ± 5.3d

*Data followed by a common letter are not significantly different ($P = 0.05$).

Table 3. Triglyceride content of blood diets

Diet	$\bar{X} \pm$ SEM ($N = 9$) (mg/dl)
FDBB-V	36.0 ± 1.3a*
FDPB-V	36.7 ± 1.0a,b
FDBB-CS	38.2 ± 1.2a,b
FBB	40.7 ± 2.9a,b
FPB	74.2 ± 2.5c
FDPB-CS	174.5 ± 2.0d

*Data followed by a common letter are not significantly different ($P = 0.05$).

Table 4. Phospholipid phosphorus content of blood diets

Diet	$\bar{X} \pm$ SEM ($N = 9$) (mg/dl)
FDBB-V	6.74 ± 0.29a*
FDPB-V	6.89 ± 0.32a,b
FDPB-CS	7.29 ± 0.21b
FDBB-CS	8.52 ± 0.15c
FBB	8.92 ± 0.20c
FPB	9.29 ± 0.10c

*Data followed by a common letter are not significantly different ($P = 0.05$).

Table 5. Phospholipid classes in blood diets

Diet	% of total phospholipid‡ SM*	PC	PI	PE†	PG
FBB	44.5a§	40.2a	0.6a	1.1a	8.4a
FDBB-CS	43.6a	39.9a	1.8b	1.3a	7.0a
FDBB-V	40.0a	30.1b	4.6b	6.5b	1.9b
FPB	24.4b	48.4c	4.1b	16.9c	1.6b
FDPB-CS	32.7c	53.7c	4.0c	2.4a	1.4b
FDPB-V	42.4a	37.7a	4.7c	6.7b	2.3b

*Lyso PE migrates the same as SM and is included in the % SM
†PS migrates the same as PE and is included in the % PE
‡Sphingomyelin = SM, phosphotidyl choline = PC, phosphotidyl inositol = PI; phosphotidyl ethanolamine = PE, phosphotidyl serine = PS phosphoglyceride = PG
§Data are means of three analyses and data followed by a common letter in same column are not significantly different ($P = 0.05$).

PS was included in the PE total because of a similar migration.

A complete fatty acid analysis of the blood diets is summarized in Table 6. The data represent an extensive effort to ascertain differences between the fatty acid content of the diets which could be correlated to fly fecundity. However, no correlation could be made, except for linolenic acid (18:3); the facundity of adults (FPB, FDPB-V and FDPB-CS in Table 1) which fed on blood diets containing lesser quantities of 18:3 was significantly greater than that of adults which fed on blood diets containing higher levels of 18:3. There is a strong negative correlation ($r = -0.8396$, $r^2 = 0.705$) between the level of 18:3 and fecundity.

DISCUSSION

Adult stable flies reared on freeze-dried blood can have a fecundity equal to that of flies reared on fresh blood. Tsetse flies have been reared on freeze-dried blood, but the fecundity was reported to be less than that of flies reared on fresh blood (DeLoach and Taher, 1983). However, in another report the fecundity of tsetse flies fed freeze-dried blood was reported to be equal to that of flies fed on fresh blood (Wetzel, 1980). Freeze-drying is known to alter certain physical and chemical characteristics of blood (DeLoach and Holman, 1983; DeLoach, 1983). Considerable effort has been devoted to improving the quality of freeze-dried blood for use in rearing tsetse flies (DeLoach and Taher, 1983; DeLoach, 1983). Additives and or processes have been found which increase the digestibility and nutritional character of the freeze-dried blood. Apparently, stable flies are not as discriminating as tsetse flies with respect to fresh or freeze-dried blood, because in tests reported here stable flies were equal in fecundity when fed either fresh or freeze-dried blood. With respect to FBB, the reproductive performance of stable flies in the 7–9 generation was unexpectedly low (Spates and DeLoach, 1985). This caused the average performance of the parental and subsequent ten generations to be lower than that found for either FDBB-V or FDBB-CS.

The nutritional superiority of pig blood over that of bovine blood has been known for tsetse flies (Langley *et al.*, 1978; DeLoach and Taher, 1983) and now is well documented for stable flies (Spates and DeLoach, 1985). A separation of blood components and recombination of porcine erythrocytes with bovine serum, and bovine erythrocytes with porcine serum, revealed that the nutritional differences were attributable to both erythrocytes and serum components (Langley *et al.*, 1978; Moloo and Pimley, 1978; DeLoach and Taher, 1983). Combinations of porcine erythrocytes with bovine serum albumin

Table 6. Fatty acid content (mg/dl) of blood diets

Fatty acids	Diets FBB	FDBB-CS	FDBB-V	FPB	FDPB-CS	FDPB-V
10:0	4.18 ± 0.18	—	—	—	—	—
12:0	0.25 ± 0.08	2.28 ± 0.24	—	0.55 ± 0.03	1.00 ± 0.41	—
13:0	1.52 ± 0.19	—	—	0.54 ± 0.04	0.39 ± 0.01	—
14:0	2.11 ± 0.21	1.05 ± 0.18	—	0.42 ± 0.02	—	—
X-1	1.98 ± 0.85	1.17 ± 0.24	1.61 ± 0.64	—	—	—
15:0	0.54 ± 0.06	0.55 ± 0.10	—	—	—	—
16:0	17.67 ± 2.50	16.29 ± 2.22	7.32 ± 0.67	22.44 ± 1.15	25.02 ± 3.95	26.36 ± 3.71
16:1	4.44 ± 0.60	3.29 ± 0.36	3.40 ± 0.35	1.18 ± 0.22	1.95 ± 0.06	2.34 ± 0.34
17:0	5.04 ± 0.55	1.73 ± 0.06	—	0.86 ± 0.19	0.79 ± 0.14	0.58 ± 0.12
X-2	0.91 ± 0.13	0.54 ± 0.07	2.48 ± 0.30	0.28 ± 0.02	0.32 ± 0.06	—
18:0	32.13 ± 4.37	26.88 ± 3.02	13.63 ± 2.36	18.29 ± 0.76	20.91 ± 2.53	20.88 ± 2.89
18:1	39.74 ± 5.25	28.56 ± 3.35	15.74 ± 1.39	37.36 ± 2.04	52.25 ± 4.88	45.92 ± 6.45
18:2	40.64 ± 5.69	42.04 ± 5.46	27.52 ± 0.42	39.29 ± 2.37	23.99 ± 2.58	22.79 ± 3.18
20:0	0.74 ± 0.15	—	3.29 ± 0.28	0.28 ± 0.01	0.57 ± 0.03	—
18:3	25.24 ± 6.10	6.19 ± 0.88	3.31 ± 0.63	1.41 ± 0.15	1.65 ± 0.09	0.65 ± 0.2[illegible]
20:1	—	—	3.06 ± 0.50	—	—	—
21:0	—	—	2.88 ± 0.05	—	—	—
22:0 + 20:4	1.90 ± 0.54	1.69 ± 0.31	2.94 ± 0.70	1.69 ± 0.08	0.39 ± 0.09	—
20:2	6.26 ± 1.45	6.32 ± 1.37	—	8.77 ± 0.7[illegible]	5.99 ± 0.71	—
X-3	3.37 ± 0.65	1.14 ± 0.29	—	—	—	—
22:1	4.84 ± 1.04	3.37 ± 1.82	2.27 ± 0.36	—	1.86 ± 0.22	8.56 ± 1.02
20:5	1.42 ± 0.09	—	—	1.76 ± 0.38	2.64 ± 0.02	—
X-4	2.02 ± 0.75	0.97 ± 0.14	1.68 ± 0.24	—	0.63 ± 0.10	1.67 ± 0.35
22:5	2.82 ± 0.95	4.24 ± 0.97	—	—	—	—
24:1	—	—	1.11 ± 0.08	1.19 ± 0.15	—	1.21 ± 0.21
22:6	1.71 ± 0.42	—	1.56 ± 1.11	—	1.69 ± 0.05	1.67 ± 0.19
X-5	—	—	21.97 ± 4.22	—	—	1.82 ± 0.35
X-6	—	—	2.18 ± 0.23	—	—	—
Total	201.47 ± 32.82	150.84 ± 17.87	117.95 ± 14.53	134.89 ± 6.98	142.47 ± 17.42	134.45 ± 12.05

The fatty acid contents of blood diets are presented as the mean ± SD of three replications. Fatty acids are presented in the same order in which their methylester derivatives eluted from the gas chromatograph.
Quantitation of the unknowns was based on the gas chromatographic response of the preceding methylester.

(BSA) and bovine erythrocytes with BSA revealed the major difference in porcine and bovine blood was in the erythrocyte component (DeLoach and Taher, 1983).

Nelson (1967a,b) evaluated phospholipid distribution in various mammalian erythrocytes and found that porcine erythrocytes contained 26.5% SM, 23.3% PC, 29.7% PE, 17.8% PS, and 2.7% other. For bovine erythrocytes, the distribution was reported to be 46.2% SM, 0% PC, 29.1% PE, 19.3% PS, and 5.4% other. Later, Buckley and Hawthorne (1972) reported that pig erythrocytes contain 9% PI. The phospholipid compositions of whole blood and freeze-dried blood reported here were expected to be different from that of the erythrocytes, due to the different lipids in serum. Since bovine erythrocytes are reported to have little or no PC, it is significant that in whole bovine blood phospholipid content is 30–40% PC. Thus, one significant difference in bovine and porcine blood is the location of the PC. This raises an important question about the nutritional superiority of porcine erythrocytes over bovine erythrocytes. In previous tests with *G. p. palpalis* (DeLoach and Taher, 1983), porcine erythrocytes mixed with 4.5% BSA was a better diet with respect to fly fecundity than bovine erythrocytes mixed with the same level of BSA. Since the two major differences in bovine and porcine erythrocytes are the proportions of SM and PC, we suggest that the locations of SM and PC in the blood components may contribute to the nutritional superiority of porcine blood.

The five major fatty acids of bovine and porcine erythrocytes are C16:0, 18:0, 18:1, 18:2 and 20:4 (Van Deenen and De Gier, 1974). Moreover, the major fatty acid fractions in the membrane of erythrocytes can be altered by changes in the diet. We found that the five major fatty acids of bovine and porcine whole blood are similar to those reported for erythrocytes. All the diets except FBB exhibited similar proportions of fatty acids. It is well known that fatty acids in the blood are a reflection of the fatty acid composition of the animal diet. A high concentration of C18:3 fatty acid in blood diets causes increased mortality and is detrimental to the reproductive capacity of tsetse flies (Moloo and Pimley, 1978). In only one diet reported here did we observe a high concentration of C18:3 (linolenic acid). FBB contained 4- to 8-fold more C18:3 than did either of the freeze-dried bovine blood diets. Also, FBB contained 15 to 38-fold more linolenic acid than did any of the pig blood diets. Perhaps the low fecundity observed in stable flies fed FBB is due to the high concentration of linolenic acid.

Our data suggest that differences in fly fecundity for porcine and bovine blood diets may be due to two factors: (1) the location of phospholipid classes—that is, in the erythrocytes or the serum; and (2) the unusually high concentration of C18:3 fatty acid in FBB. Further studies of these factors are needed to determine their relative importance.

REFERENCES

Bauer B. and Aigner H. (1978) *In vitro* maintenance of *Glossina palpalis palpalis* (Robineau-Desvoidy) (Diptera: Glossinidae). *Bull. Ent. Res.* **68**, 393–400.

Bridges A. C. and Spates G. E. (1983) Larval medium for the stable fly, *Stomoxys calcitrans* (L.). *Southwest. Ent.* **8**, 6–10.

Buckley J. T. and Hawthorne J. N. (1972) Erythrocyte membrane polyphosphoinositide metabolism and the regulations of calcium binding. *J. biol. Chem.* **247**, 7218–7213.

DeLoach J. R. (1983) Sonication of reconstituted freeze-dried bovine and porcine blood: Effect on fecundity and pupal weights of the tsetse fly. *Comp. Biochem. Physiol.* **76A**, 47–49.

DeLoach J. R. and Holman G. M. (1983) An amino acid analysis of freeze-dried bovine and porcine blood used for *in vitro* rearing of *Glossina palpalis palpalis* (Rob.-Desv.). *Comp. Biochem. Physiol.* **75A**, 499–502.

DeLoach J. R. and Spates G. E. (1984) Hemoglobin and albumin diet for adult *Stomoxys calcitrans* and *Glossina palpalis palpalis*. *Southwest. Ent.* **9**, 28–34.

DeLoach J. R. and Taher M. (1983) Investigations on development of an artificial diet for *in vitro* rearing of *Glossina palpalis palpalis* (Diptera: Glossinidae) *J. Econ. Ent.* **76**, 1112–1117.

Duck-Chong C. G. (1978) A rapid sensitive method for determining phospholipid phosphorus involving digestion with magnesium nitrate. *Lipids* **14**, 492–497.

Folch J., Lees M. and Slogne Staneley G. H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J. biol. Chem.* **226**, 497–509.

Hoffman R. A. (1968) The stable fly *Stomoxys calcitrans* (Linnaeus): Biology and behavior studies. Ph.D. dissertation, Oklahoma State University, Stillwater, OK.

Ivie G. W. and Wright J. E. (1978) The fate of diflubenzuron in the stable fly and house fly. *J. Agric. Food Chem.* **26**, 90–94.

Langley P. A., Pimley R. W., Mews A. R. and Flood M. E. T. (1978) Effect of diet composition on feeding digestion and reproduction in *Glossina morsitans*. *J. Insect Physiol.* **24**, 233–238.

Moloo S. K. and Pimley R. W. (1978) Nutritional studies in the development of *in vitro* feeding techniques for *Glossina morsitans*. *J. Insect. Physiol.* **24**, 491–497.

Nelson G. J. (1967a) Lipid composition of erythrocytes in various mammalian species. *Biochem. biophys. Acta* **144**, 221–232.

Nelson G. J. (1967b) Composition of neutral lipids from erythrocytes of common mammals. *J. Lipid Res.* **8**, 374–379.

Spates G. E. (1979) Fecundity of the stable fly: Effect of soybean trypsin inhibitors and phospholipase A inhibitor on the fecundity. *Ann. Ent. Soc. Am.* **72**, 845–849.

Spates G. E. and DeLoach J. R. (1985) Reproductive performance of adult stable flies (Diptera: Muscidae) when fed fresh or reconstituted freeze-dried bovine or porcine blood. *J. Econ. Ent.* **78**, 856–859.

Van Deenen L. L. M. and De Gier J. (1974) Lipids of the red cell membrane. In *The Red Blood Cell*, 2nd edn, Vol. 7, pp. 147–211. Academic Press, New York.

Wetzel H. (1978) The use of deep freezer, stored bovine blood for *in vitro* feeding of tsetse flies. *Z. Parasitenk* **57**, 163–168.

Wetzel H. (1980) The use of freeze-dried blood in the membrane feeding of tsetse flies (*Glossina p. palpalis* Diptera: Glossinidae). *Tropenmed. Parasitol.* **31**, 259–274.

Reproductive Performance of Adult Stable Flies (Diptera: Muscidae) When Fed Fresh or Reconstituted, Freeze-dried Bovine or Porcine Blood[1]

G. E. SPATES AND J. R. DELOACH

Veterinary Toxicology and Entomology Research Laboratory,
U.S. Department of Agriculture, Agricultural Research Service,
College Station, Texas 77841

J. Econ. Entomol. 78: 856–859 (1985)

ABSTRACT The reproductive performances were compared of adult stable flies, *Stomoxys calcitrans* (L.), fed fresh porcine or bovine blood or reconstituted, freeze-dried porcine or bovine blood. Adult stable flies fed fresh or reconstituted, freeze-dried porcine blood oviposited significantly more eggs than adults fed fresh or freeze-dried bovine blood. No decline in fecundity was observed for adults maintained for 10 successive generations on fresh porcine blood or on reconstituted, freeze-dried porcine or bovine blood. However, fecundity of adults maintained on fresh, heparinized bovine blood declined in later generations. The data provide evidence that stable fly adults can be maintained successfully on reconstituted, freeze-dried blood, and that porcine blood is nutritionally superior to the blood of cattle.

MALE AND FEMALE stable flies, *Stomoxys calcitrans* (L.), are obligate blood-feeders, and feed on a wide variety of hosts (Newstead 1906, Bishopp 1913, Hafez and Gamal-Eddin 1959, Anderson and Tempelis 1970, Monty 1972, DuToit 1975, LaBrecque et al. 1975, Howell et al. 1978). Presently, most laboratory colonies are maintained on fresh, nonsterile, citrated bovine blood (Doty 1937, Bailey et al. 1975), which can be obtained from a local abattoir, or they can be maintained by feeding the adults on small animals or livestock. No genuine attempts have been made to determine alternate sources of nutrients for adults because of this ease of maintenance.

Recently it has been reported that adult tsetse flies can be maintained on freeze-dried bovine or porcine blood. Wetzel (1979, 1980) reported that *Glossina palpalis palpalis* (Robineau-Desvoidy) fed freeze-dried blood performed as well as flies fed in vivo on the guinea pig with respect to fecundity and offspring weights; however, there was a decline in pupal weights through successive reproductive cycles. This decline in pupal weights may have been due to elevated rearing room temperatures, according to observations of DeLoach and Taher (1983). Therefore, they reassessed the reproductive performance (fecundity and pupal weights) of adult tsetse flies fed freeze-dried blood alone or fed bovine serum albumin (BSA) or cholesterol. DeLoach and Taher (1983) observed that the reproductive performance of adults fed freeze-dried blood was lower in comparison to that of adults fed on guinea pigs. The addition of BSA to freeze-dried blood improved the reproductive performance, whereas the addition of cholesterol had a negative effect.

No studies have been conducted to determine whether or not stable flies can be maintained on freeze-dried blood or on fresh porcine blood. If adult stable flies can be successfully maintained on freeze-dried or porcine blood, the dependency on fresh, citrated bovine blood would be eliminated, especially for mass-rearing methods. The use of freeze-dried blood would also enable investigators to maintain colonies of stable flies in areas where fresh bovine blood is not readily obtainable, and alleviate the need for maintaining laboratory animals or livestock for feeding the parasites.

This study was undertaken to determine if adult stable flies could be maintained on freeze-dried blood and to evaluate their reproductive performance by monitoring fecundity and egg hatch. The results are reported herein.

Materials and Methods

Stable flies used in these studies were taken from a colony maintained at our laboratory since 1969. Detailed conditions for colony maintenance and egg collection have been described (Ivie and Wright 1978). The larval rearing method was modified in 1979, as described by Bridges and Spates (1983).

Blood diets used in these studies were as follows. Freeze-dried bovine blood (FDBB [CS]) and freeze-dried porcine blood (FDPB [CS]) were prepared at College Station, Tex. Freeze-dried bovine blood (FDBB [Vien.]) and freeze-dried porcine blood (FDPB [Vien.]) were prepared in Vienna, Austria. Fresh, heparinized bovine (FHB) and porcine blood (FHP) was from College Station. Blood used for freeze-drying was obtained from slaughterhouses

[1] This article reports the results of research only. Mention of a proprietary product does not constitute endorsement or recommendation for its use by USDA.

Rec'd in SCI APR 10 1985

cc BOSTICK
CDIE

Table 1. Fecundity of female stable flies fed on fresh whole blood and reconstituted, freeze-dried whole blood and subsequent egg hatch

Blood meal	Eggs/day/♀	Hatch (%)	Total eggs counted
FHP	56.7 ± 0.9a	88.6 ± 2.3	34,100
FDPB [Vien.]	51.3 ± 3.4a	94.7 ± 1.1	28,900
FDPB [CS]	50.9 ± 0.7a	92.8 ± 1.7	28,400
FDBB [CS]	42.3 ± 0.5b	94.3 ± 1.8	24,100
FHB	39.2 ± 1.0b	93.3 ± 1.1	22,100
FDBB [Vien.]	37.5 ± 0.8b	95.9 ± 1.1	21,100

Means within a column followed by the same letter are not significantly different ($P < 0.05$ for fecundity data, $n = 4$, $\omega = 7.9$; Tukey's multiple comparison procedure). No significant difference in percent egg hatch.

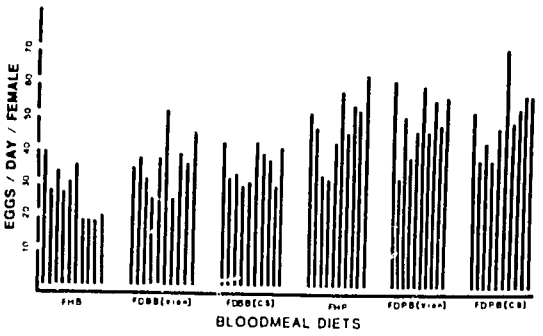

Fig. 1. Fecundity of adult stable flies maintained on reconstituted, freeze-dried, and fresh blood for 10 consecutive generations.

in Vienna, Austria, and from College Station, Tex. Blood was freeze-dried on the dates collected according to the procedures of Wetzel (1979). Freeze-dried blood was reconstituted in distilled water at a water-to-blood ratio of 4.1:1. Enough blood was reconstituted to feed each generation of adults, and kept frozen in small culture tubes (1.5-ml aliquots) at −20°C. Before feeding, blood was thawed and warmed to 30°C. Flies were allowed to feed to satiation from dental rolls saturated with blood (Ivie and Wright 1978).

To establish base-line fecundity data for the performance of stable flies on freeze-dried blood, two groups of newly emerged adults (5 males, 10 females) for each diet were randomly selected and placed in small plastic cups (9 cm diam by 7 cm high) with screened tops and bottoms. They were fed 24 h later and daily thereafter for 12 days. On day 5, the males were removed and discarded. The females were placed in clean cages and oviposition was initiated the same day. Thereafter, females were allowed to oviposit daily for the next 7 days and mortality of females recorded. This procedure was replicated four times. To determine the performance of stable flies on freeze-dried blood, three groups of adults (6 males, 12 females) for each diet were chosen from the stock colony and fed the respective diets for 10 consecutive generations. Feeding and handling of adults was as previously stated, with one exception: 100 eggs were taken from the second or third oviposition and placed on rearing media for the succeeding generation. When adults of this generation emerged, they were placed on the same diet as were succeeding generations. During generations 7 to 10, some adults from each generation were fed citrated bovine blood (the food source for the original colony) and their fecundity was ascertained. In these studies, fecundity was measured by counting all eggs oviposited; hatchability of all eggs was determined 48 h after each oviposition.

Data were subjected to an analysis of variance using a repeated measure design (Winer 1971). The conservative degrees of freedom test (as demonstrated in Winer [1971]) was used to judge significance because of possible variance-covariance matrix assumption resolutions associated with repeated measured designs.

Results and Discussion

The base-line reproductive performance and percentage egg hatch from stable flies fed reconstituted or fresh, heparinized blood are summarized in Table 1. Fecundity of adults that fed on FHP was significantly higher than the fecundity of adults that fed on FHB ($P < 0.05$). Females that fed on porcine blood oviposited an average of 53 eggs per day and the average for females that fed on bovine blood was about 39.7 per day. The fecundity of females fed bovine blood in this study compares favorably with previous reports for this colony of stable flies. Spates (1979) reported that this colony oviposited an average of 603 eggs during a 16-day (extrapolated data) period (7 to 22 days postemergence). Females used in this study oviposited an average of 303 eggs per female in an 8-day period (5 to 13 days postemergence). There were no significant differences in the percentage egg hatch between adults fed on any of the various types of blood. It may be noted that females fed fresh porcine blood had the highest fecundity but had the lowest egg hatch.

Ten successive generations of adults were maintained on reconstituted, freeze-dried, and fresh blood with varying degrees of success. The fecundity for 10 generations of females is shown in Fig. 1, and a summary of fecundity and egg hatch is given in Table 2. Surprisingly, females maintained on FHB exhibited the poorest reproductive performance, particularly for generations 6 to 10. The mean number of eggs oviposited per day per female with FHB was 50% less than on reconstituted porcine or fresh porcine blood. In fact, stable flies maintained on porcine blood had significantly higher fecundities than did flies maintained on bovine blood (Table 2).

Table 3 summarizes data on the reproductive performance of females taken from generations 7 to 10, when fed citrated bovine blood. All females, except those that had been maintained on fresh

Table 2. Summary of statistical analysis of the fecundity and egg hatch data on female stable flies that were maintained for 10 consecutive generations on reconstituted, freeze-dried blood or fresh heparinized blood

Blood meal	Mean	
	Eggs/day/♀	Egg hatch (%)
FDPB [CS]	50.0a	93.6a
FHP	49.4a	87.8a
FDPB [Vien.]	48.3a	91.0a
FDBB [Vien.]	36.8b	87.1a
FDBB [CS]	35.4b	91.2a
FHB	27.1c	74.2b

Means within a column followed by the same letter are not significantly different ($P < 0.05$: for fecundity data, $n = 33$, $\omega = 6.35$; for percent hatch, $n = 33$, $\omega = 6.07$; Tukey's multiple comparison procedure).

Table 3. Reproductive performance of adult stable flies fed on six different diets when subsequently fed citrated bovine blood

Blood meal diets of parent generations[a]	Mean[b]	
	Eggs/day/♀	Egg hatch (%)
FDPB [Vien.]	39.9 ± 4.28a	84.89 ± 11.22a
FHP	38.28 ± 6.72ab	82.30 ± 9.02a
FDPB [CS]	33.62 ± 4.15b	91.00 ± 5.98a
FDBB [CS]	33.96 ± 6.70b	83.72 ± 9.99a
FDBB [Vien.]	30.22 ± 9.46bc	89.05 ± 7.52a
FHB	23.84 ± 5.08d	73.60 ± 13.89b

Means within a column followed by the same letter are not significantly different ($P < 0.05$: for fecundity data, $n = 12$, $\omega = 4.74$; for percent hatch, $n = 12$, $\omega = 8.20$; Tukey's multiple comparison procedure).

[a] Adults were maintained for 10 successive generations on the respective blood meal diets; some of the adult progeny of the 7th to 10th generations were re-evaluated by feeding them citrated blood collected from the local slaughterhouse.

[b] Means of two groups of 12 females from the 7th to 10th generations

bovine blood, averaged more than 30 eggs per day. The highest reproductive performance involved females that had been maintained on reconstituted, freeze-dried porcine blood from Vienna, and the lowest reproductive performance was shown by the group that had been maintained on fresh bovine blood. Egg hatch exceeded 82% for all groups, except the one that had been maintained on fresh bovine blood.

Our data indicate that stable flies can be maintained on freeze-dried blood without any apparent loss in fecundity or egg hatch. Freeze-dried blood used in our study was taken from random lots that DeLoach and Taher (1983) prepared and used for their tsetse fly studies. However, our study was conducted at a later date. Although DeLoach and Taher (1983) observed that tsetse flies fed freeze-dried blood had reduced fecundity and smaller offspring in comparison to tsetse flies that fed in vivo on guinea pigs, our study revealed that the fecundity and egg hatch of stable flies fed freeze-dried blood was equal to or higher than the fecundity and egg hatch of adults fed fresh bovine blood (Tables 1 and 2). J. R. DeLoach and S. E. Kunz (personal communication) were unable to maintain horn flies on freeze-dried blood because of high adult mortality. From previous studies with horn flies, stable flies, and tsetse flies, it is evident that blood meal diets that are adequate for one blood-feeding dipteran may be insufficient or totally inadequate for a related species.

Also, our data indicate that porcine blood is nutritionally superior to bovine blood because 1) female stable flies that were fed the porcine blood initially oviposited an average of 25% more eggs; 2) after 10 successive generations on the respective blood diets, females fed porcine blood oviposited an average of 32% more eggs; and 3) of the females from generations 7 to 10 re-evaluated on citrated bovine blood, those from the groups fed porcine blood oviposited an average of 21% more eggs than did those from the groups fed bovine blood. It is well known that porcine blood is nutritionally superior to bovine blood as tsetse fly diet and several suggestions have been made to explain this phenomenon. Langley et al. (1978) suggested that the nutritional superiority of porcine blood is due to a property of the blood itself and not to the quantities of blood ingested by the tsetse fly. Moloo and Pimley (1978) suggested that the inferiority of bovine blood for in vitro feeding of the tsetse fly is due to a small deficit of proteins, since additives such as linoleic acid, amino acids, or vitamins were ineffective in overcoming this deficit. DeLoach and Taher (1983) believed that the difference in response of *Glossina* spp. to porcine and bovine blood may be due to lipid composition.

The reproductive performance of adult stable flies that were fed reconstituted, freeze-dried porcine and bovine blood exceeded our expectations; their fecundities were equal to or greater than those previously reported for this strain of stable flies (Spates 1979). However, the decline in fecundity of the later generations of flies fed fresh, heparinized bovine blood was not expected (Fig. 1). Egg hatchability of these generations also declined (unpublished data). The maximum reproductive capacity of the stable fly has not been determined, but conceivably, maximum fecundity may have been attained with the fresh porcine blood.

Acknowledgment

We are indebted to W. F. Fisher (U.S. Livestock Insects Laboratory, Kerrville, Tex.) for conducting the statistical analyses. We also wish to thank J. Coleman and C. Ayala for their technical assistance. This work was partially supported by the Agency for International Development.

References Cited

Anderson, J. R., and C. H. Tempelis. 1970. Precipitin test identification of blood meals of *Stomoxys calcitrans* (L.) caught on California poultry ranches,

and observations of digestion rates of bovine and citrated human blood. J. Med. Entomol. 7: 223–229.

Bailey, D. L., T. L. Whitfield, and G. C. LaBrecque. 1975. Laboratory biology and techniques for mass producing the stable fly, *Stomoxys calcitrans* (L.) (Diptera: Muscidae). Ibid. 12: 189–193.

Bishopp, F. C. 1913. The stable fly (*Stomoxys calcitrans* L.), an imported livestock pest. J. Econ. Entomol. 6: 112–126.

Bridges, A. C., and G. E. Spates. 1983. Larval medium for the stable fly, *Stomoxys calcitrans* (L.). Southwest. Entomol. 8: 6–10.

DeLoach, J. R., and M. Taher. 1983. Investigations on development of an artificial diet for *in vitro* rearing of *Glossina palpalis palpalis* (Diptera: Glossinidae). J. Econ. Entomol. 76: 1112–1117.

Doty, A. E. 1937. Convenient method of rearing the stable fly. Ibid. 30: 367–369.

DuToit, C. D. G. 1975. Reproductive capacity and longevity of stable flies maintained on different kinds of blood. J. S. Afr. Vet. Assoc. 46: 345–347.

Hafez, M., and F. M. Gamal-Eddin. 1959. On the feeding habits of *Stomoxys calcitrans* L. and *sitiens* Rond., with special reference to their biting cycle in nature. Bull. Soc. Entomol. Egypt 43: 291–301.

Howell, C. J., J. B. Walker, and E. M. Neville. 1978. Ticks, mites and insects infesting domestic animals in South Africa. Part I. Descriptions and biology. Repub. S. Afr. Dep. Agric. Tech. Serv. Sci. Bull. No. 393.

Ivie, G. W., and J. E. Wright. 1978. The fate of diflubenzuron in the stable fly and house fly. J. Agric. Food Chem. 26: 90–94.

LaBrecque, G. C., D. E. Weidhaas, and T. L. Whitfield. 1975. Graphic models as intermediate steps to computerized simulations of stable fly populations. Mosq. News 35: 316–321.

Langley, D. A., R. W. Pimley, A. R. Mews, and M. E. Flood. 1978. Effects of diet composition on feeding, digestion, and reproduction in *Glossina morsitans*. J. Insect Physiol. 24: 233–238.

Moloo, S. K., and R. W. Pimley. 1978. Nutritional studies in the development of *in vitro* feeding techniques for *Glossina morsitans*. Ibid. 24: 491–497.

Monty, J. 1972. A review of the stable fly problem in Mauritius. Rev. Agric. Sucr. Ile Maurice 51: 13–29.

Newstead, R. 1906. On the life-history of *Stomoxys calcitrans* Linn. J. Econ. Biol. 1: 157–166.

Spates, G. E. 1979. Fecundity of the stable fly: effect of soybean trypsin inhibitor and phospholipase A inhibitor on the fecundity. Ann. Entomol. Soc. Am. 72: 845–849.

Wetzel, H. 1979. Artificial membrane for *in vitro* feeding of piercing-sucking arthropods. Entomol. Exp. Appl. 25: 117–119.

1980. The use of freeze-dried blood in the membrane feeding of tsetse flies (*Glossina p. palpalis*, Diptera: Muscidae). Tropenmed. Parasitol. 31: 259–274.

Winer, B. J. 1971. Statistical principles in experimental design, 2nd ed. McGraw-Hill, New York.

Received for publication 7 August 1985; accepted 25 March 1985.

Comp. Biochem. Physiol. Vol. 83A, No. 1, pp. 133–139, 1986
Printed in Great Britain

0300-9629/86 $3.00 + 0.00
© 1986 Pergamon Press Ltd

2. H-04

STUDIES ON THE BIOCHEMICAL BASIS OF THE NUTRITIONAL QUALITY OF TSETSE FLY DIETS

J. P. KABAYO, J. R. DELOACH*, G. E. SPATES*, G. M. HOLMAN* and G. M. KAPATSA*

Entomology Unit, FAO/IAEA Agricultural Biotechnology Laboratory, Seibersdorf, P.O. Box 100, A-1400 Vienna, Austria and *USDA Veterinary Toxicology and Entomology Research Laboratory, P.O. Drawer GE, College Station, Texas 77841, USA

(*Received* 7 *May* 1985)

Abstract—1. Batches of freeze-dried pig and cow blood, whose nutritional value to *G. p. palpalis* ranged from low to near optimum, were analysed for amino acid, triglyceride and cholesterol content.

2. The results of the chemical analyses were compared with the nutritional quality parameters observed when each batch of blood was fed to *G. p. palpalis* in an attempt to establish a chemical basis for the nutritional quality of diets for *Glossina*.

3. In general, those pig or cow blood diets that had a higher nutritional quality also had a significantly higher amino acid content than the suboptimal diets.

4. There were significant differences between the triglyceride and cholesterol content of pig and cow blood, with pig blood having more triglyceride, but less cholesterol than bovine blood.

5. There was no apparent correlation between the triglyceride and cholesterol content and the nutritional quality of blood.

INTRODUCTION

In the past attempts have been made to improve the efficiency and flexibility of artificial methods of feeding *Glossina*. The flies can now be fed *in vitro* on a variety of blood products or derivatives, including fresh defibrinated blood (Mews *et al.*, 1977), reconstituted freeze-dried blood (Bauer and Wetzel, 1976) and, more recently, synthetic diets (Kabayo *et al.*, 1985). The variability and differences in the nutritional quality of blood from different sources have generated: (i) concern amongst those wishing to standardize or optimize productivity in tsetse fly mass-rearing operations, and (ii) a great deal of interest among scientists in search of a quantitative and qualitative correlation between the chemical composition of diets and their nutritional value to tsetse flies.

The nutritional superiority of pig blood to cow blood observed for most species of tsetse (e.g. Mews *et al.*, 1977) and the known nutritional quality variability between different batches of blood have necessitated that quality control tests be conducted on small groups of flies by which the suitability of each batch of blood is ascertained before the blood can be used to feed main colony flies (Van der Vloedt, in preparation). Attempts have been made to determine the nutritional value of specific blood components (Kabayo and Langley, 1985), but apart from the indirect evidence for the nutritional requirement of lipids deduced from deletion studies (Kabayo, 1982; Kabayo and Langley, 1985) and the comparative amino acid analysis of fresh (Moloo and Pimley, 1978) or freeze-dried (DeLoach and Holman, 1983) bovine and porcine bloods, there is no information available on the relationship between nutritional adequacy and specific chemical components of tsetse diets. Such information would be useful for establishing a biochemical basis of nutritional quality, for formulating synthetic diets and for improving suboptimal diets.

Protein is the main nutritional component in vertebrate blood, the exclusive source of food for tsetse flies, amounting to about 90% of its dry wt (Altman and Dittmer, 1961). It is probable that the amino acid composition of tsetse diets has a direct influence on the nutritional properties of blood products of which they form the major components. In addition, since the nutritional importance of lipids for the reproductive physiology of tsetse has been suggested (Kabayo, 1982; Kabayo and Langley, 1985), it is possible that lipids too could be indices of nutritional quality. In the present investigation attempts were made to relate the amino acid, triglyceride and cholesterol composition of batches of freeze-dried blood to the differences in nutritional quality observed when the different batches of blood were fed to *Glossina palpalis palpalis*.

MATERIAL AND METHODS

Diets and feeding experiments

Different batches of freeze-dried pig and cow blood (collected from a commercial abattoir, defibrinated and freeze-dried on separate occasions) were each reconstituted in distilled water at a concentration of 24.39%, irradiated (100 krad), supplemented with ATP* and fed to groups of *G. p. palpalis* females. For each batch of blood tested, two groups, each of 15 freshly emerged female *G. p. palpalis* were set up and fed five times a week for 25 days. All handling, maintenance conditions and rearing procedures were as

*ATP, adenosine triphosphate disodium salt, Merck, was added to the diets as a phagostimulant at a final concentration of 10^{-3} mol l^{-1} before the flies were fed.

Rec'd in SCI APR 10 1990

cc BUSTIA
CDIE

60

previously described (Van der Vloedt, 1982). The number, mean weight of puparia deposited and the survival of flies fed each batch of blood were recorded.

Amino acid analysis

Total amino acids in each of the freeze-dried blood samples were analysed according to standard procedures for a Beckman Model 121 amino acid analyser. Tryptophan was not determined.

Lipid extraction and analysis

With the exception of acetic acid and diethylether all solvents were redistilled before use. Silica gel (20 × 20 cm, 250 μm thick) Type H thin-layer chromatography plates (Analtech, Newark, DW) were used. Prior to use, the TLC plates were activated by heating in an oven at 110 C for 40 min. TLC and gas chromatography standards were purchased from Supelco, Inc., Bellenfonte, Pennsylvania, except triglycerides, cholesterol and cholesterol esters, which were obtained from Sigma Chemical Co., St. Louis, Missouri. Reagent kits for the determination of triglycerides and cholesterol were obtained from Dow Diagnostics, Indianapolis, Indiana and Worthington Diagnostics, Freehold, New Jersey, respectively.

Lipid extractions were carried out on two 15 ml aliquots of each of the reconstituted (20%, w/v, in distilled water) freeze-dried pig and cow blood samples using ice-cold methanol and chloroform according to the method of Spates and DeLoach (1984). The extracted lipids were subsequently used in quantitative estimations of triglycerides and cholesterol and in analyses using thin-layer and gas chromatography techniques.

Triglycerides and cholesterol were estimated by enzymatic procedures (using Dow Diagnostic and Worthington Diagnostic reagent kits, respectively) on a Gilford System 3500 Analyser. Duplicate samples each of 100 and 200 μl aliquots of the chloroform:methanol (2:1, v/v) extracts were evaporated to dryness under nitrogen and each sample residue was taken up in 200 μl isopropanol and subsequently analysed separately for triglyceride and cholesterol. Standards containing known amounts of triglyceride of cholesterol, dissolved in isopropanol were similarly analysed and used to calibrate the values obtained for the extracted lipid samples.

Thirty microliters of each of the lipid extracts and standards were separately streaked onto activated Silica gel thin-layer plates under a stream of nitrogen. The plates were eluted with *n*-hexane:diethylether:acetic acid (60:40:2, v/v). The separated lipid classes were stained with iodine vapour and then photographed.

Six hundred microliters of each of the lipid extracts was mixed with in 1.5 ml 10% potassium hydroxide and hydrolysed by heating on a waterbath for 30 min. The hydrolysate was neutralized with a 4.5 m 1N HCl following which the entire mixture was extracted twice with 4 ml petroleum ether. The petroleum ether extracts were evaporated to dryness under nitrogen, then the residue was taken up in 1 ml petroleum ether, mixed with 0.5 ml diazo-methane and left to stand for 20 min. The methylated fatty acid esters were dissolved in petroleum ether and analysed by gas chromatography on a Tracor MT 220 GC instrument equipped with a 1.83 × 6.3 cm glass column packed with 10% SP-2330 (100/120 mesh) supelcoport (Supelco, Inc.).

Haemoglobin and physical parameters

Each diet was analysed for haemoglobin by the cyanomehaemoglobin method with a Coulter Electronics Haemoglobinometer (Hialeah, Floride). The osmotic pressure of each reconstituted diet was determined with a vapour pressure osmometer (Wescor Inc., Logan, Utah). Total particle counts and particle sizes were determined with a Coulter counter equipped with a computerized channel analyser (Coulter Electronics, Hialeah, Florida).

Table 1. Performance of *G. p. palpalis* fed different batches of freeze-dried pig and cow blood

Parameter	Pig blood samples						Cow blood samples					
	1	2	3	4	5	6	1	2	3	4	5	6
Nutritional quality factor	0.70	0.73	1.0	1.08	1.01	1.0	0.41	0.72	1.0	1.01	1.01	1.06
% survival	76.7	83.3	100	79.3	90	100	62.1	66.77	96.7	79.3	73.3	86.7
No. pupae per initial female	0.23	0.23	0.41	0.76	0.63	0.47	0.07	0.10	0.37	0.67	0.63	0.70
Mean puparial weight mg (± SD)	27.26 (1.67)	27.06 (2.03)	29.38 (1.98)	30.14 (3.11)	30.92 (1.86)	30.06 (3.02)	26.5 (2.45)	27.02 (2.31)	27.34 (2.99)	27.89 (2.54)	26.97 (3.21)	28.31 (2.28)

Data for 25 days.

Table 2. Triglyceride and cholesterol content of different batches of freeze-dried pig and cow blood

	Pig blood samples						Cow blood samples					
Parameter	1	2	3	4	5	6	1	2	3	4	5	6
Triglyceride (mg g)	3.62	3.89	4.11	4.50	3.28	4.78	2.83	2.92	2.24	2.60	3.15	2.65
Cholesterol (mg g)	4.55	4.66	5.61	5.69	4.76	4.97	6.39	5.84	6.24	7.68	7.50	6.43

RESULTS

The performance of groups of *G. p. palpalis* females fed different batches of freeze-dried pig and cow blood is shown in Table 1. Flies fed on pig blood in general had heavier pupae than those fed cow blood. There were variations in the fecundity (pupae deposited per initial female) and in the mean puparial weights for flies fed different batches of either pig or cow blood.

The triglyceride and cholesterol content of the different batches of pig and cow blood is shown in

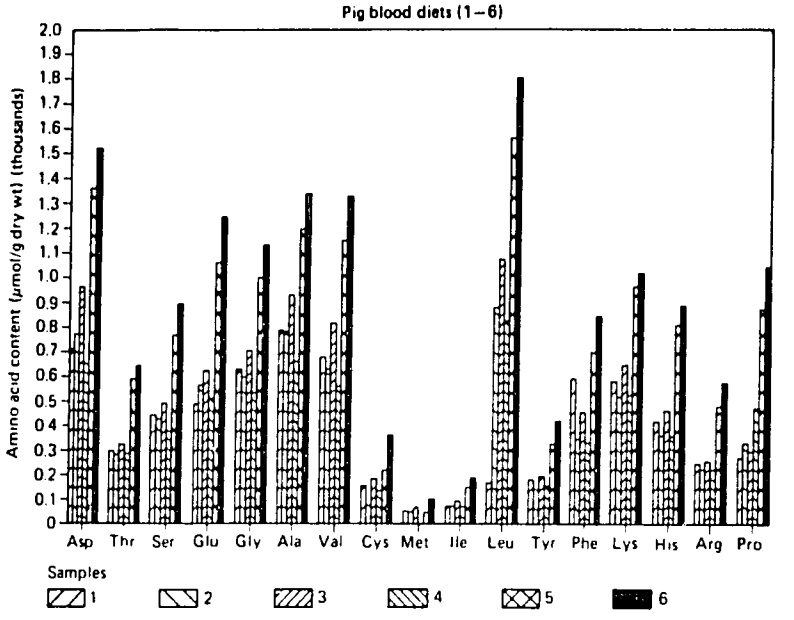

Fig. 1. Amino acid composition of pig blood diets 1–6. In general the nutritional quality of these diets increased in the order 1–6.

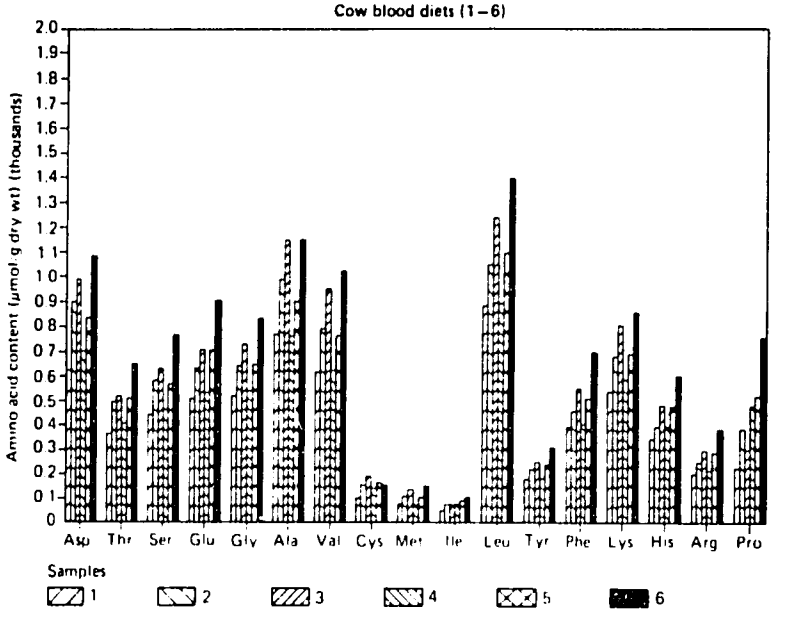

Fig. 2. Amino acid composition of cow blood diets 1–6. In general the nutritional quality of these diets increased in the order 1–6.

Table 3 (a) Total amino acid content of pig blood samples (μmol/g dry weight)

Asp	Thr	Ser	Glu	Gly	Ala	Val	1/2 Cys	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Pro
714.51	299.14	445.31	585.67	628.28	787.76	682.96	157.88	54.87	79.10	968.19	182.62	592.54	583.59	419.85	248.87	272.50
771.58	284.64	429.39	564.03	590.25	780.68	633.48	72.78	48.26	73.85	880.51	151.93	350.40	520.96	367.13	228.58	333.30
967.88	328.97	496.89	625.76	705.17	932.43	818.34	186.29	71.34	95.29	1075.0	198.51	453.46	648.11	465.19	251.64	300.0
650.25	288.83	382.48	514.54	633.79	662.76	562.84	155.18		76.80	826.89	157.49	333.93	528.07	362.98	225.57	479.00
1357.70	594.51	769.97	1062.10	999.26	1197.60	1148.30	220.86	47.30	153.66	1561.60	332.41	704.0	967.58	809.49	482.50	875.0
1523.10	651.58	898.88	1240.0	1128.5	1333.6	1326.1	362.94	103.15	191.1	1805.5	424.96	851.4	1018.2	886.2	579.11	1041.5

Table 3 (b) Total amino acid content of cow blood samples (μmol/g dry weight)

Amino acids		Asp	Thr	Ser	Glu	Gly	Ala	Val	1/2 Cys	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Pro
Cow blood samples	1	626.01	369.26	446.36	510.41	516.30	771.34	616.19	104.99	80.17	49.72	884.21	178.38	399.46	539.04	344.82	199.23	227.00
	2	898.45	475.12	585.71	634.14	638.91	988.47	791.33	155.23	110.87	62.46	1051.20	217.99	462.86	676.32	395.64	247.35	388.85
	3	995.76	515.70	634.76	708.81	727.70	1148.20	949.97	189.75	135.56	76.80	1241.80	250.59	553.95	802.60	483.02	297.10	300.00
	4	676.55	406.37	466.69	534.40	538.90	759.59	625.84	141.22	76.19	77.68	894.35	184.66	407.02	585.62	359.17	214.36	479.00
	5	834.36	511.32	578.68	704.12	649.42	898.50	761.46	162.4	99.64	91.12	1096.80	238.66	507.92	688.0	477.40	290.09	583.33
	6	1086.5	649.48	766.08	903.26	832.81	1152.3	1022.2	145.66	155.25	105.68	1397.1	310.23	699.3	856.7	602.8	381.81	750.0

Table 2. There are significant differences (t-test $P < 0.05$) between the triglyceride and cholesterol content of pig and cow bloods, with pig blood containing more triglyceride but less cholesterol than cow blood. There is no apparent correlation between lipid content (triglyceride or cholesterol) and the nutritional quality of the different blood batches.

The total amino acid content of the different freeze-dried blood samples is shown in Tables 3a and 3b and in Figs 1 and 2. There were only slight differences between the amino acid content of pig and cow blood batches, with pig blood having only marginally more aspartic acid, isoleucine, half-cystine, leucine and proline. In either pig or cow blood batches of blood there was a general tendency for those blood batches with high nutritional quality parameters to have a higher amino acid content.

The results of the gas chromatographic analysis of the total lipids extracted from the different blood batches are shown in Table 4, and Figs 3 and 4. There is a significantly higher proportion of the longer chain fatty acid components in cow blood than in pig blood. There is no correlation between the nutritional quality properties of the batches of blood and their fatty acid composition.

The results of thin-layer chromatography of the total lipids extracted are shown in Fig. 5. Pig blood samples appear to have a higher content of tri-, di- and monoglycerides than cow blood samples.

Haemoglobin concentrations in all the pig and cow blood batches were quite constant (Table 5). The osmotic pressure was not different between cow and pig blood. Particle counts were slightly higher for cow blood than for pig blood. Particle size was the same for both bloods.

DISCUSSION

In order to understand the nutritional requirements of insects, consideration must be given to the relationship between the chemical composition and nutritional quality of their diets, more especially when the insects in question are specialized, restricted feeders such as species of *Glossina*, which feed exclusively on vertebrate blood. Although the general composition of mammalian blood is known (Altman and Dittmer, 1961), it is also known to vary a great deal depending on the age, sex, physiological states, etc. of donor animals. Further variation in the composition of blood used as *in vitro* diets of *Glossina* may originate during collection, storage or handling, from chemical or microbiological contaminants and from modification by physical factors. It is likely that such variations in the composition of blood may be the cause of the variability observed in the nutritional quality of blood from different sources.

The present results attempt to correlate differences between chemical composition differences and nutritional quality differences observed for different blood batches and are suggestive of the possibility that the nutritional quality of *Glossina* diets could be chemically defined. DeLoach and Spates (1984) used amino acid analysis data in attempt to explain the nutritional value differences between haemoglobin, serum albumin and whole blood as diets for *Stomoxys calcitrans* and *G. p. palpalis*. In the present

Table 4. % Areas of GC peaks for pig and cow blood lipids at different retention times

Retention times ± 0.4 min		Chemical structure	Pig blood samples 2	3	4	5	Cow blood samples 2	3	4	5
	14.25	C14	0.311	0.987	0.997	0.260	0.520	0.247	1.898	1.458
	15.19		—	0.479	0.587	0.293	0.337	0.429	1.008	0.531
	16.44		0.102	0.139	0.166		—	—	—	—
	16.93		—					0.525	0.394	0.659
	17.69		0.123	0.356	0.389	0.184	0.219	0.426	0.609	0.678
	18.96		—	—			0.189	0.309	0.146	0.205
	20.22	C16	18.323	18.779	16.135	18.818	12.249	7.653	7.841	7.168
	21.84	C16:1	1.460	1.732	1.485	0.997	2.341	2.34	2.448	1.925
	22.71	C18:0		0.821	0.668	0.504	0.520	0.929	0.938	0.927
	24.25		0.601	0.305	0.301	0.199	0.237	0.581	0.442	0.505
% peak area	25.36		15.076	14.021	12.613	15.462	14.670	14.373	14.869	14.048
	26.79	C19:0								
		C18:1	32.151	30.094	27.591	28.918	23.964	21.231	21.308	17.430
	28.91	C18:2	19.992	21.270	21.389	20.161	22.417	25.461	24.398	27.411
	30.34	C20:0	0.221	0.174	0.205	0.147	0.182	0.191	0.173	0.122
	31.30	C18:3								
		C20:1	0.569	0.750	0.866	0.587	1.803	2.522	2.098	1.899
	33.95		0.053	0.189	0.364	0.342	0.383	0.549	0.280	0.435
	35.18	C22:0								
		C20:4	0.691	0.966	0.972	0.959	0.876	1.303	1.726	1.189
	36.56	C22:1								
		C20:5	7.657	5.914	6.623	6.512	6.357	4.628	4.207	3.900
	37.57		—	—	—	—	0.461	0.634	0.707	0.787
	39.31		0.449	0.479	1.592	1.264	0.893	1.166	1.057	1.055
	42.25	C22:4								
		C22:5	0.601	0.586	0.757	0.662	2.715	5.349	3.903	10.179
	46.57		0.851	0.986	1.163	0.922	1.304	0.462	0.324	0.294
	48.47		0.577	0.584	0.410	0.768	—	—	—	—
	50.33		—	—	0.541	0.293	1.513	2.539	1.231	1.055
	55.08	C22:6	ND	0.437	2.419	1.369	4.962	6.072	7.194	5.223

% areas calculated for peaks corresponding to retention times of 14–55 min. Other peaks not considered. Values for pig and cow blood samples 1 and 6 not included.

studies, diets (pig or cow blood) which were shown to have high nutritional quality in general also had high amino acid content. There is extensive literature on the nutritional evaluation of proteins or protein-containing feeds for mammals (e.g. Munroe and Allison, 1964; Campton and Harris, 1970), but there is little information on evaluation of proteins in foods of insects. Bursell and Taylor (1980) measured the energy values of vertebrate blood as a means of assessing equivalence between metabolic inputs and outputs, but they did not compare the energy values of blood samples with different nutritional qualities.

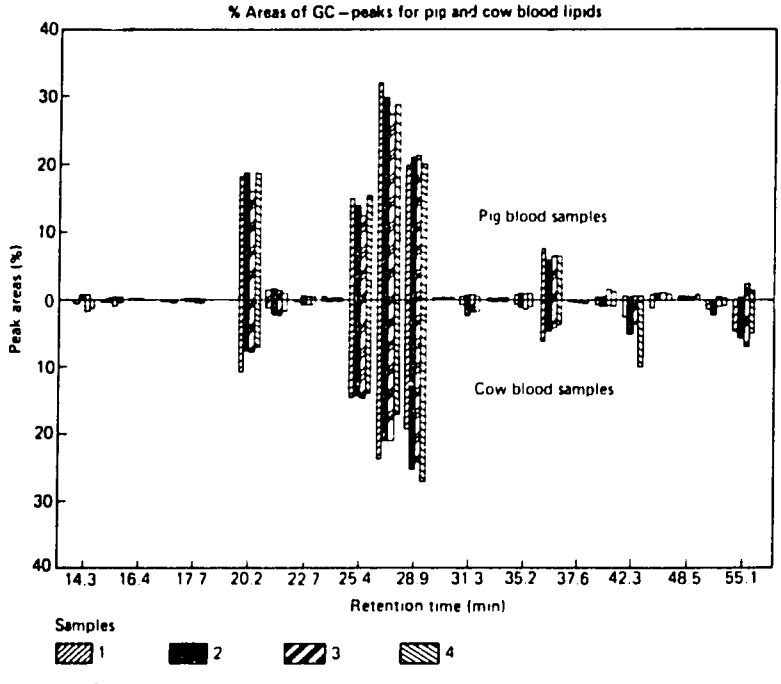

Fig. 3. % Areas of gas chromatography peaks for pig and cow blood lipids at various retention times.

Table 5. Haemoglobin and physical parameters of freeze-dried pig and cow bloods

Freeze-dried blood samples	Haemoglobin g %	Osmotic pressure mOsm/kg	Particle count (10^{-}) ml	Particle size 10^{-15} l
Pig blood 1	13.2	282	1.96	41.9
Pig blood 2	14.0	310	1.82	45.6
Pig blood 3	14.4	318	2.07	39.6
Pig blood 4	16.1	303	2.62	52.3
Pig blood 5	14.1	293	1.97	41.4
Pig blood 6	14.3	316	1.67	41.0
Cow blood 1	13.0	316	2.44	50.6
Cow blood 2	13.9	283	3.03	39.1
Cow blood 3	13.8	293	2.53	38.0
Cow blood 4	14.5	283	2.84	53.8
Cow blood 5	13.3	312	2.98	34.7
Cow blood 6	11.9	307	2.49	36.4

The "protein score" procedure used by Davis (1972) and Rock (1972) to assess the nutritional value of feeds for *Tenebrio molitor* and *Argyrotaenia velutinana*, respectively was criticized by McGinnis and Kasting (1972) because each insect species has a unique protein requirement that is optimum for its growth, development and reproduction. The nutritional value of proteins resides not only in their amino acid composition, but also in the quantities of each amino acid liberated during protein digestion that is subsequently absorbed. The differences in amino acid content between the batches of blood may be due to differences in total protein concentration and also to any differences in the solubility and digestibility of the samples. Kabayo *et al.* (1985) improved the nutritional quality of a commercial preparation of haemoglobin by an initial treatment to remove insoluble material; and DeLoach (1983) reported increased fecundity and puparial weights of *G. p. palpalis* fed reconstituted freeze-dried blood whose solubility was enhanced by sonication.

There were significant lipid (triglyceride and cholesterol) differences between pig and cow blood, but none that could be correlated to the nutritional differences either between pig and cow bloods or to differences in the batches of each blood type. The nutritional importance of lipids for tsetse flies have been suggested by the results of various investigations (Kabayo, 1982; Kabayo and Langley, 1985), and for *Stomoxys calcitrans* (Spates and DeLoach, 1986). Because of the difficulties to render lipids soluble in aqueous dietary media, the nutritional importance of lipids has so far been inferred from diet deletion studies. However, when synthetic diets incorporating varying concentrations of liposome-borne cholesterol were fed to *G. p. palpalis*, a higher mortality was associated with higher concentrations of cholesterol (Kabayo and Taher, unpublished observations), and flies fed freeze-dried bovine blood supplemented with cholesterol were shown to have lower fecundity than those fed control diets (DeLoach and Taher, 1983).

Acknowledgements—Our thanks are due to Drs A. M. V. Van der Vloedt, R. E. Gingrich and D. A. Lindquist and to Messrs M. Taher and L. Mayr, all of the International Atomic Energy Agency, P.O. Box 100, A-1400 Vienna, Austria, for their advice or help in this work. We are also grateful to Dr R. T. Mayer for encouragement in this work and to Mr C. Ayala and Ms E. J. Coleman of the USDA Veterinary Toxicology and Entomology Laboratory, College Station, Texas, for technical assistance.

REFERENCES

Altman P. L. and Dittmer D. S. (1961) Blood and other body fluids. *Fed. Proc. Fed. Amer. Soc. exp. Biol. M.D., U.S.A.*

Bauer B. and Wetzel H. (1976) A new membrane for feeding *Glossina morsitans*. *Bull. Entomol. Res.* **65**, 563–565.

Bursell E. B. and Taylor P. (1980) An energy budget for *Glossina*. *Bull. Entomol. Res.* **70**, 187–196.

Campton E. W. and Harris L. E. (1970) *Applied Animal Nutrition*, pp. 57–103. Freeman, San Francisco.

Davis G. R. F. (1972) Redefining diets for optimal performance. In *Insect and Mite Nutrition. Significance and Implication in Ecology and Pest Management* (Edited by Rodriguez J. R.), pp. 171–181. North Holland, Amsterdam.

DeLoach J. R. (1983) Sonication of reconstituted freeze-dried bovine and porcine blood. Effect on fecundity and puparial weights of the tsetse fly. *Comp. Biochem. Physiol.* **76A**, 47–49.

DeLoach J. R. and Spates G. E. (1984) Haemoglobin and albumin diet for adult *Stomoxys calcitrans* and *Glossina palpalis palpalis*. *Southwest. Entomol.* **9**, 28–34.

DeLoach J. R. and Holman G. M. (1983) An amino acid analysis of freeze-dried bovine and porcine blood used for *in vitro* rearing of *Glossina palpalis palpalis*. *Comp. Biochem. Physiol.* **75A**, 499–502.

DeLoach J. R. and Taher M. (1983) Investigations on development of an artificial diet for *in vitro* rearing of *Glossina palpalis palpalis*. *J. Econ. Entomol.* **76**, 1112–1117.

Kabayo J. P. (1982) The nature of the nutritional importance of serum albumin to *Glossina morsitans*. *J. Insect. Physiol.* **28**, 917–923.

Kabayo J. P., Taher M. and Van der Vloedt A. M. V. (1985) Synthetic diet for *Glossina*. *Bull. Entomol. Res.* (in press)

Kabayo J. P. and Langley P. A. (1985) The nutritional importance of individual blood components to *G. m. morsitans*. *J. Insect Physiol.* **31**, 619–624.

McGinnis A. J. and Kasting R. (1972) Protein evaluation in insect diets. In *Insect and Mite Nutrition. Significance and Implication in Ecology and Pest Management* (Edited by Rodriguez J. G.), pp. 57–71. North Holland, Amsterdam.

Mews A. R., Langley P. A., Pimley R. W. and Flood M. E. (1977) Large-scale rearing of tsetse flies (*Glossina* spp.) in the absence of a living host. *Bull. Entomol. Res.* **67**, 119–128.

Moloo S. K. and Pimley R. W. (1978) Nutritional studies in the development of *in vitro* feeding techniques for *G. morsitans*. *J. Insect Physiol.* **24**, 491–497.

Munroe H. W. and Allison J. B. (1964) *Mammalian Protein Metabolism*, Vol. 2. Academic Press, New York.

Rock G. C. (1972) Optimal proportions of dietary amino acids. In *Insect and Mite Nutrition. Significance and Implication in Ecology and Pest Management* (Edited

by Rodriguez J. G.), pp. 183-197. North Holland, Amsterdam.

Spates G. E. and DeLoach J. R. (1986) A lipid analysis of freeze-dried blood and the reproductivity of *Stomoxys calcitrans* fed on freeze-dried blood. *Comp. Biochem. Physiol.* (in press).

Van der Vloedt A. M. V. (1982) Recent advances in tsetse mass-rearing with particular reference to *Glossina palpalis palpalis* fed *in vitro* on guinea pigs. In *Sterile Insect Technique*. IAEA-SM-255/11, pp. 223-253.