# THE COMPOSITION OF BAT MILK:

# A CHEMICAL ANALYSIS

by

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STATEMENT BY AUTHOR

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APPROVAL BY THESIS DIRECTOR

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Wm. J. McCauley — April 22, 1963

W. J. McCauley, Associate Professor of Zoology — Date

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# INTRODUCTION

Considerable interest has been generated in the composition of previously unstudied mammalian milks in an attempt to better define phylogenetic relationships. The absence of lactose in the milk of the California Sea Lion, Zalophus californianus (Pilson and Kelly, 1962) might help to set Pinnipedia apart from the Carnivora which have lactose present in their milk (Evans, 1959). One recent study by Sowls et al., (1961) on the composition of the milk of the collared peccary, Tayassu tajacu, indicated distinct differences when compared to domestic sow milk. Electrophoretic analyses of the milk proteins of a variety of mammals representing forty species and eight orders was conducted (Sloan et al., 1961). Sloan and his co-workers have made a distinction between three electrophoretic patterns of casein: First, a 'primitive' pattern having a single peak corresponding to cow $\alpha$-casein and appearing among some rodents, lagomorphs and marsupials. The second type has a prominent, slow-moving peak corresponding to cow $\beta$-casein and a faster-moving fraction which adsorbs greatly

to the paper. This latter type is supposed to be 'more advanced' and is found in some carnivores and primates. The third, and 'most advanced' pattern found in Bos and most other mammals has a slow-moving $\beta$ -casein peak and a faster-moving $\alpha$ -casein peak. In addition, Bos shows a third component, $\gamma$ -casein which is very slow-moving and not prominent in other artiodactyls. These representative patterns are illustrated in Figure 2, page 18.

Representative species from two different families of bats which utilize distinctly different types of food were selected for this study: Leptonycteris nivalis of the family Phyllostomatidae, a pollen and nectar feeding bat (Beatty, 1955), and Tadarida brasiliensis of the family Molossidae, an insectivorous bat (Ross, 1961).

Since the two species are separable on a nutritional basis, differences in the composition of the milks may be due to dietary differences. Following the definition of dietary influences, other biochemical similarities in the milks may be attributable to familial relationships.

There has been no previously published analysis of the milk of any member of the Order Chiroptera.

## MATERIALS AND METHODS

*Leptonycteris nivalis* and *Tadarida brasiliensis* are both fairly large bats and readily available in maternity colonies near Tucson, Arizona. Female *L. nivalis* were collected from a maternity colony located in Colossal Cave, Vail, Arizona. The animals were collected in hand nets inside a tunnel entrance to the bat room of the cave after the tunnel had been barricaded. The collections were made between 25 May, 1962 and 23 June, 1962. The post partum lactating animals were retained for milking. The collections were made between the hours of 11:00 P.M. and 2:00 A.M. after the animals had been feeding and were returning to the cave. Female *T. brasiliensis* were taken from a maternity colony under a railroad bridge 4.5 miles south of Continental, Arizona, between 10 July, 1962 and 13 July, 1962. They were taken in hand nets in the early morning hours after the return from feeding.

Several rather elaborate devices have been devised for collecting milk from small laboratory animals (Cox and Mueller, 1937; Mueller, 1939; Kahler, 1941-2; Temple

and Kon, 1937), but the complexity of most of these methods compared with the small amount of sampling necessary in this study made a simpler means more practical. After some trial and error in attempting to obtain milk from lactating mice, and after being advised that manual expression following injection of oxytocic hormone (Pitocin, Parke-Davis) facilitated obtaining milk from Microtus pennsylvanicus (R. E. Sloan, personal communication), the following method was decided upon.

The animals were anesthetized lightly with sodium nembutal (1/20 dilution of 3/4 gm/mg) 10 to 15 hours after capture. It was determined that approximately 0.1 milliliter of the nembutal would lightly anesthetize L. nivalis while approximately 0.03 to 0.05 milliliter was used for T. brasiliensis. This was administered simultaneously with an injection of less than 0.1 milliliter Pitocin (Parke-Davis). The mammaries were bathed with warm water before milking and were gently pressed with thumb and index finger to extrude the milk in droplets. These droplets were drawn into a hemocytometer pipette. The samples from several animals were pooled and transferred to a weighed vial. Since the maximum amount of milk obtained from any one animal did

not exceed 0.3 milliliter, pooling of the samples was necessary. The pooled samples ranged in weight from 0.3 to 0.8 gram. To aid in the preservation of samples a small drop of 10% formalin was added and the vials refrigerated (Official Methods of Analysis of the A.O.A.C., 1950). In most cases bovine milk samples were analyzed previous to the bat milk and compared with known results to insure proper technique. Where desirable the bovine milk was also analyzed as a simultaneous control.

The pH of each sample was determined on a Beckmen model g pH meter immediately following milking.

The density of the milk samples was determined by the use of micro-pipettes (corrected for water volumes). The density of the samples was determined by four trials with a control sample of bovine milk. All measurements were made at 22°C and weighed on a Sartorius-Werke balance within 30 seconds after removal from a constant temperature bath (Niederl and Niederl, 1942).

Total solids and ash content were determined on whole milk samples following the procedure outlined by Official Methods of Analysis, A.O.A.C. (9th Edition, 1960) with some modifications. A portion of each sample was

weighed in a porcelain crucible. The crucible was previously heated in an oven at 100°C and cooled in a desiccator. The weight of the samples ranged from 0.2 g to 0.5 g for chiropteran milk and 1.0 g for the bovine control. For ash determination the residue was ignited in an electric muffle furnace at a temperature not exceeding 550°C for approximately 8 hours and again cooled in a desiccator and weighed. The percentages of total solids and the ash content were calculated.

The determination of percent lactose followed the method used by Marier and Boulet (1959). The colorimeter used was the Bausch and Lomb Spectronic 20 with a wave length setting of 490 . A standard curve was plotted for each trial and the percent lactose was read from this curve.

Percent protein was determined following the Micro-Kjeldahl procedure of Ma and Zuazaga (1942). The distillate was titrated with 0.018 N HCl to the disappearance of the blue-green color.

In addition to the bovine milk samples as controls, a sample of ammonium sulfate was analyzed and the experimental results compared with the calculated amount of nitrogen

present. This method for determining nitrogen was selected as superior to other techniques for Micro-Kjeldahl determination for the following reasons: 1. It allows a direct titration of the distillate, eliminating any back titration. 2. The standard solutions are limited to hydrochloric acid only. 3. The mixed indicator gives a sharper end point (Ma and Zuazaga, 1941).

The samples used in electrophoretic analyses of proteins and chromatographic analyses of sugars were prepared from single whole milk samples. The method of preparation followed that used by Sowls et al. (1961). The sample of whole milk was centrifuged and the upper fat layer was removed. The casein protein fraction was precipitated out of solution by adding 0.1 N acetic acid to a pH of approximately 4.5. The casein was removed by centrifugation and washed several times with water. It was then freeze-dried and weighed.

The solution remaining after casein removal was dialyzed exhaustively against water at a reduced temperature (5-10°C) to remove the sugars. The dialyzed portion contained the soluble whey proteins which were freeze-dried and weighed. The dialysate was retained for chromatographic analysis of sugars and was deionized using the

Baird and Tatlock Model 300 desalting apparatus.

For electrophoresis the freeze-dried proteins were dissolved to approximately 5.0% solutions (8.0% solutions suggested by R. E. Sloan, 1962, were not feasible with the small samples used). The electrophoretic studies were carried out using a Spinco Model R apparatus. Caseins were run in veronal buffer at pH 8.6, ionic strength 0.05 for 16 hours at 5.0°C. Whey proteins were done in veronal buffer at pH 8.6, ionic strength 0.075 for 16 hours at room temperature. A prepared sample of bovine casein or whey was used as a control on each run by comparison to established patterns. The whey dialysate was subjected to descending paper chromatography using a solvent of ethyl acetate, pyridine and water in the ratio 5:2:7. The solvent front was allowed to move beyond the paper to facilitate separation and, after drying, the chromatogram was sprayed with a mixture of aniline and an n-butanol solution of phthalic acid. The chromatogram was developed in a drying oven at 105°C for 5 minutes (McFarren, et al. 1951). An ultraviolet light was used to scan the developed chromatograms.

## RESULTS

In the following section the experimental results have been tabulated in a comparative manner to show the similarities and differences between the milks of *T. brasiliensis* and *L. nivalis*. The results of the bovine milk sample controls are included in most of the tables and figures. Table 6, page 22 is a summary of the chemical and physical properties determined in this study and compared with other results.

The pH determinations made on the bat milk samples are summarized in table 1. As indicated, the pH of the milk from *L. nivalis* was higher than that of *T. brasiliensis* although both were acidic.

The results of the specific gravity (or density) determinations of whole milk samples are tabulated in table 2.

It is evident from table 3 that milk of *T. brasiliensis* shows a significantly higher percent total solids than the milk of either *L. nivalis* or *Bos*.

The standard curve in Figure 1, page 13 is a composite of the curves plotted for lactose determinations.

TABLE 1. Results of pH determinations on milk of *L. nivalis* and *T. brasiliensis*

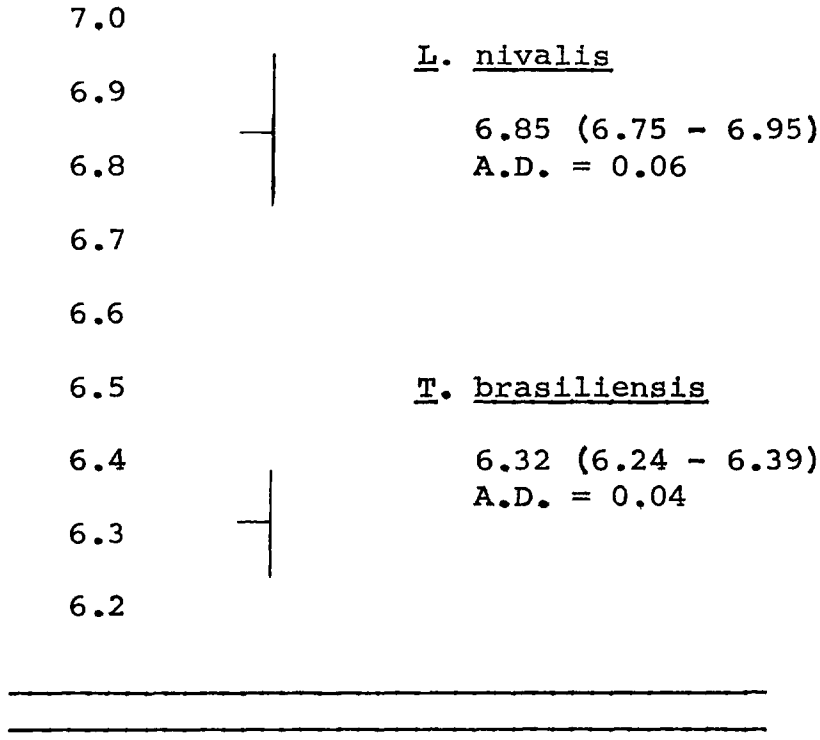

7.0
6.9
6.8
6.7
6.6
6.5
6.4
6.3
6.2
L. nivalis
6.85 (6.75 - 6.95)
A.D. = 0.06
T. brasiliensis
6.32 (6.24 - 6.39)
A.D. = 0.04

TABLE 2. Results of specific gravity determinations on milks of *Bos*, *L. nivalis* and *T. brasiliensis*.

A. D. = average deviation of mean.

Bos

| Wt. Sample (g) | Specific Gravity |
|---|---|
| 0.0223 | 1.037 |
| 0.0222 | 1.032 |

L. nivalis

| | |
|---|---|
| 0.0223 | 1.014 |
| 0.0224 | 1.004 |
| 0.0228 | 1.036 |
| 0.0231 | 1.050 |

Mean = 1.026
A.D. = 0.017

T. brasiliensis

| | |
|---|---|
| 0.0216 | 1.004 |
| 0.0220 | 1.023 |
| 0.0221 | 1.028 |
| 0.0220 | 1.023 |

Mean = 1.034
A.D. = 0.002

TABLE 3. Percent total solids and percent ash.

| | Wt. Sample (g) | % Total Solids | % Ash |
|---|---|---|---|
| *L. nivalis* | 0.3949 | 12.1 | 0.63 |
| *T. brasiliensis* | 0.2851 | 29.7 | |
| | 0.1915 | 39.0 | 0.73 |
| *Bos* | 1.4576 | 12.3 | 0.67 |
| | 0.5570 | 10.5 | 0.66 |

FIGURE 1. Lactose standard curve.

Bausch and Lomb Spectronic 20

Wave length = 490 millimicrons

Standard lactose = 0.005 mg/ml.

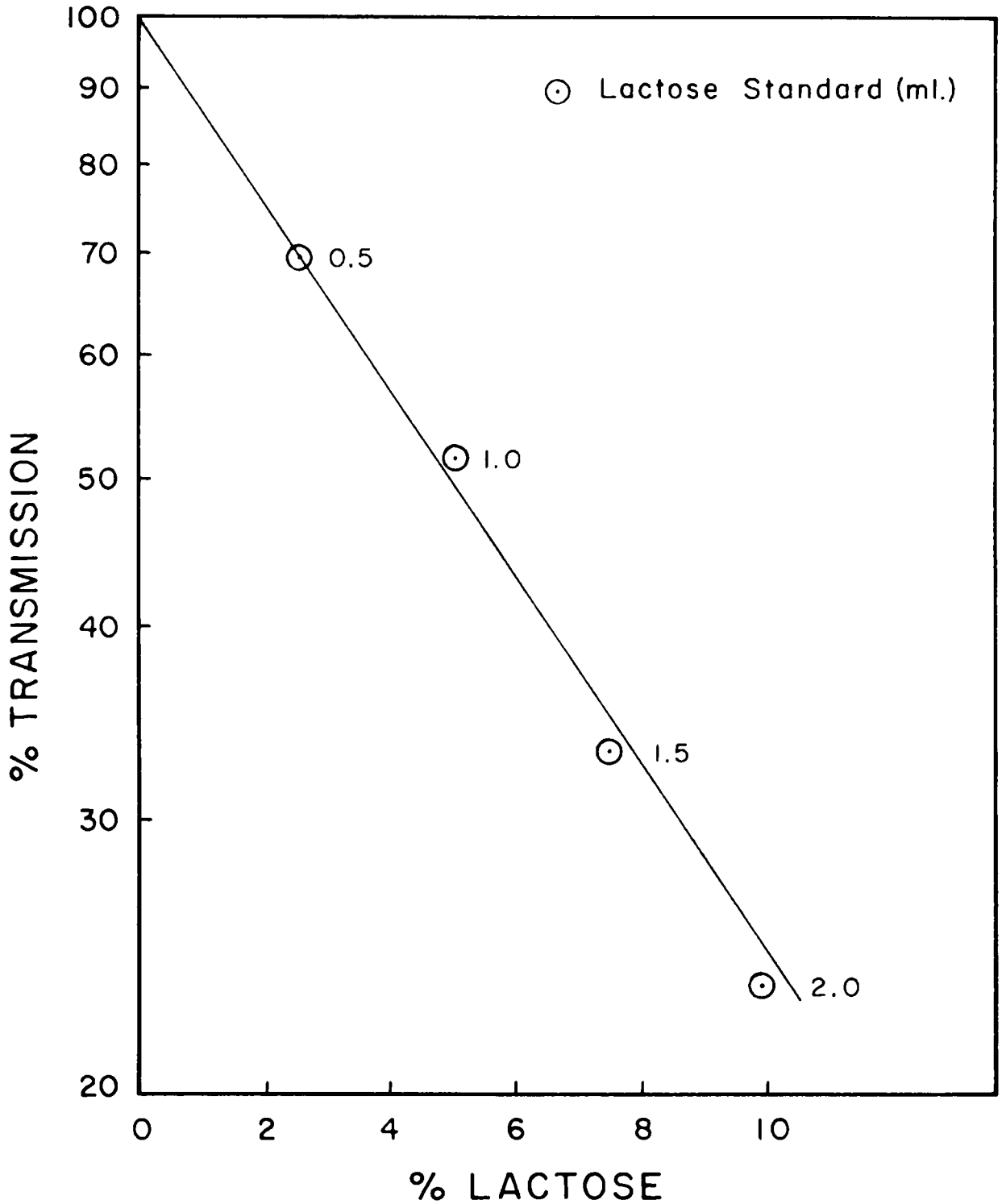

100
90
80
70
60
50
40
30
20
% TRANSMISSION
⊙ Lactose Standard (ml.)
0.5
1.0
1.5
2.0
0
2
4
6
8
10
% LACTOSE

TABLE 4. Percent lactose (above) and standard curve (below). Values given are averages for percent transmission (%T) determined and for percent lactose (%L) as read from plotted standard curve. A.D. = average deviation from mean.

| | Bos | L. nivalis | T. brasiliensis |
|---|---|---|---|
| Sample Number | 5 | 5 | 6 |
| Mean % T | 59.3 | 47.6 | 59.9 |
| A.D. | 2.98 | 2.50 | 3.38 |
| Mean % L | 4.48 | 5.39 | 3.70 |
| A.D. | 0.42 | 0.47 | 0.48 |

Standard Curve

| (ml S L) | 0.0 | 0.5 | 1.0 | 1.5 | 2.0 |
|---|---|---|---|---|---|
| Sample Number | 4 | 4 | 4 | 2 | 4 |
| Mean % T | 100 | 69.7 | 51.6 | 33.2 | 23.7 |
| A.D. | | 4.07 | 2.75 | 1.95 | 1.62 |

The mean values and average deviations for the plotted points are given in table 4, page 14 together with a summary of the lactose percentages.

In determining percent protein the Micro-Kjeldahl technique of Ma and Zuazaga (1941) as previously described was used. The results are given in table 5, page 16.

Calculations of percentages were made following volumetric determinations by the formulae:

$$\text{Wt. Nitrogen (mg)} = \text{ml acid} \times \text{normality acid} \times \text{equiv. wt. Nitrogen}$$

$$\frac{\text{mg Nitrogen}}{\text{Wt. Sample (mg)}} = \text{Total Nitrogen}$$

$$\% \text{ Protein} = \% \text{ Total N} \times 6.38$$

The errors involved in the determinations include weighing a small sample, titration to a visible end point, and columetric standardization of the acid. There are errors also in the calculations due to the assumption that the percent nitrogen in protein is relatively constant (usually 15.38%). A five percent error may be assumed due to non-protein nitrogen.

These errors can be minimized by carrying out other determinations, a procedure not allowable in this

TABLE 5. Micro-Kjeldahl determination of nitrogen and calculated percent protein. Mean values are given for *Bos* milk samples. (% protein determined using the factor 6.38)

| | No. Samples | Wt. Sample (g) | Nitrogen (mg) | Nitrogen (%) | Protein (%) | A.D. |
|---|---|---|---|---|---|---|
| Bos | | | | | | |
| | 8 | 0.506 | 2.425 | 0.480 | 3.057 | 0.082 |
| T. brasiliensis | | 0.2621 | 4.321 | 1.65 | 10.53 | |
| | 2 | 0.2870 | 5.235 | 1.82 | 11.61 | |
| L. nivalis | | | | | | |
| | 1 | 0.3169 | 2.171 | 0.685 | 4.37 | |

| | Wt. Sample (g) | Calculated Nitrogen (mg) | Determined Nitrogen (mg) | % Error |
|---|---|---|---|---|
| | 0.206 | 4.3672 | 4.3365 | 0.47 |
| Ammonium Sulfate | 0.013 | 2.7772 | 2.6117 | 0.59 |

case due to the limited size and number of samples. To help insure minimum error in technique two controls were used. These controls consisted of analytical determination of nitrogen from known samples of ammonium sulfate and simultaneous analyses of bovine milk samples. I feel that the significance is of comparative rather than absolute values and since the same error is inherent in all determinations and is not of an experimental nature, it may be ignored.

In examining the electrophoretic patterns of milk one should bear in mind that each peak may possibly represent a group of proteins rather than a single one. For some animal species these peaks have been identified for specific protein groups while for others a comparison is made with the known protein groups on the basis of migratory rate. The casein patterns are shown in figure 3 and the whey protein patterns in figure 4. Figure 2 shows for comparison three general types of casein proteins given in the literature (Sloan *et al.*, 1961).

The casein patterns of *T. brasiliensis* and *L. nivalis* show all three components of bovine casein quite distinctly.

FIGURE 2. Casein electrophoretic patterns. Units in cm.

1. *Didelphis*; 'primitive' pattern
2. *Mephitis*; 'more advanced' pattern
3. *Bos*; 'most advanced' pattern

(from Sloan *et al.*, 1961)

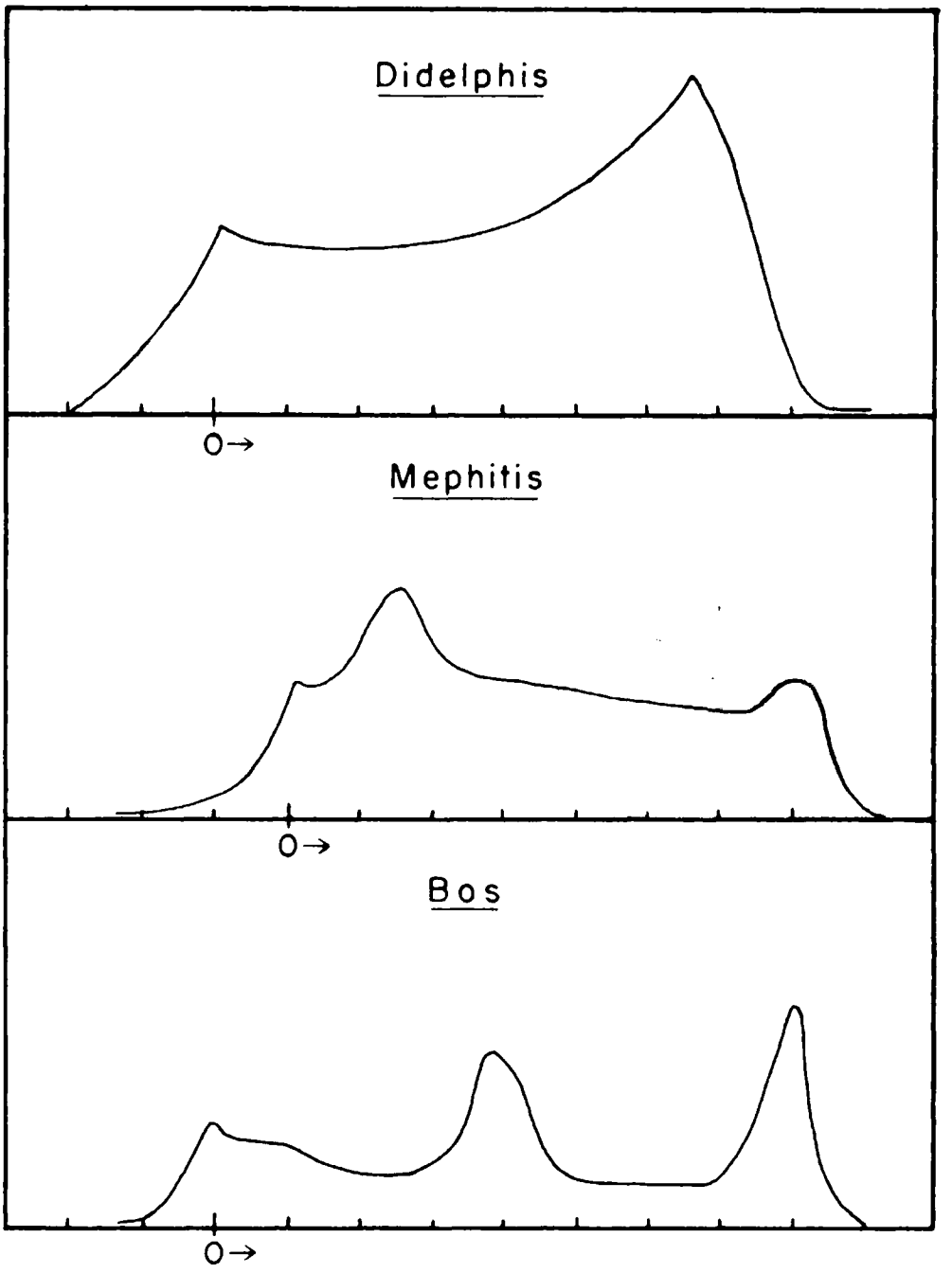

Didelphis
0→
Mephitis
0→
Bos
0→

FIGURE 3. Casein electrophoretic patterns.

Spinco Model R
Veronal pH 8.6
Ionic strength 0.05
Temp. = 5°C
Time 16 hrs.

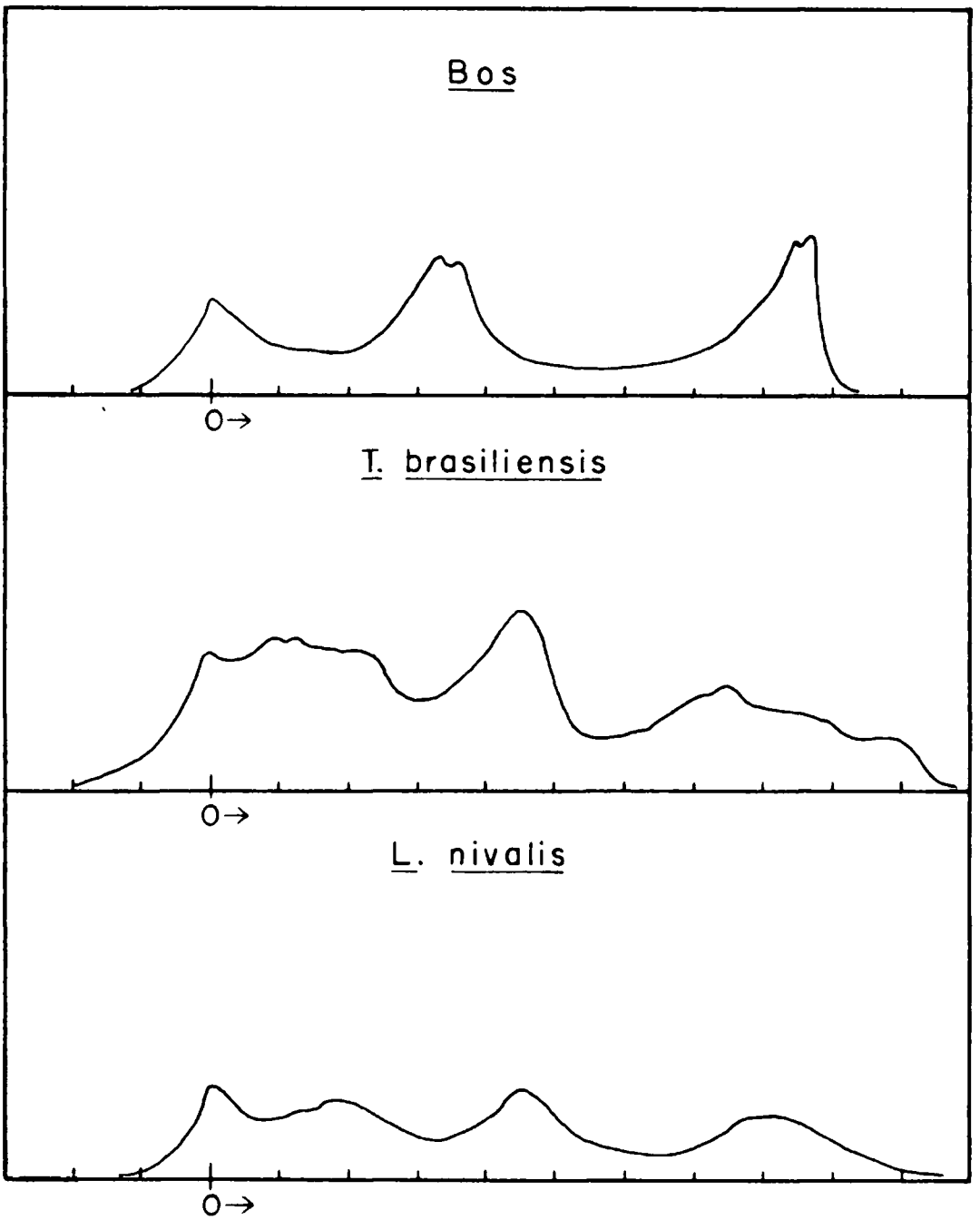
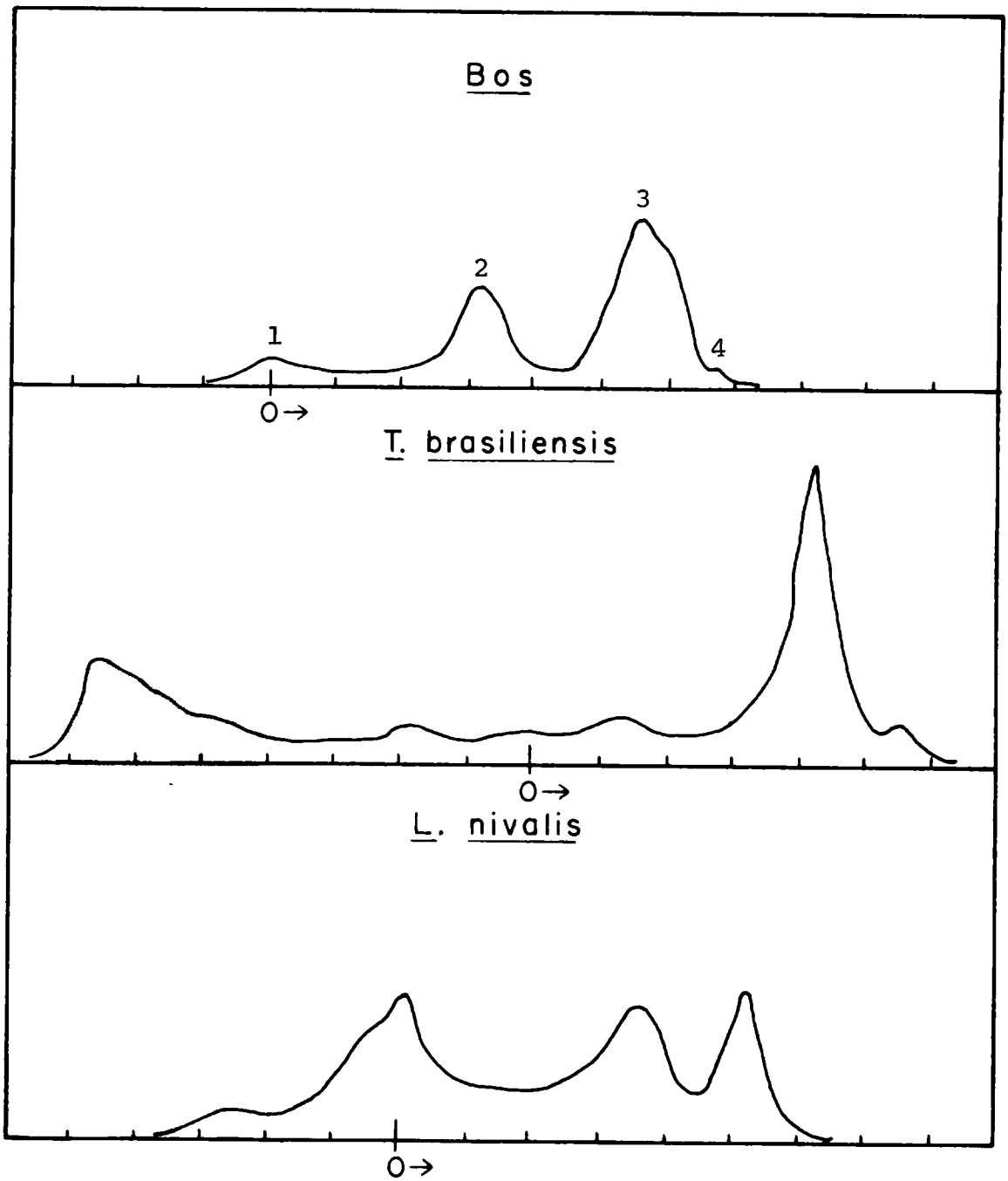

Bos
0→
T. brasiliensis
0→
L. nivalis
0→

FIGURE 4. Paper electrophoretic patterns - whey protein.

Spinco Model R
Veronal pH 8.6
Ionic strength 0.075
Temp. = 25°C
Time 16 hrs.

Bos:

1. = immune globulins
2. = a-lactalbumin
3. = b-lactoglobulin
4. = bovine blood serum albumin


Bos
1
2
3
4
0→
T. brasiliensis
0→
L. nivalis
0→

Both show a pronounced bovine $\beta$ -casein component and, in addition, T. brasiliensis displays a rather complex pattern of the slowest-moving and fastest-moving fractions.

Chromatographic runs were made on the whey dialysate to determine the presence of lactose or other sugar components. It had been suggested (R. E. Sloan, 1962, personal communication) that possibly other sugars such as glucose, galactose or various polysaccharides of pentoses are present in the milk of primitive theria. The results of the chromatography confirmed the presence of lactose but did not demonstrate the presence of glucose or galactose (which were run as known control samples), nor were any other spots present for which there were no control samples.

Table 6 is a summary of the chemical and physical properties of bat and cow milk as determined in this study (columns C,D, and E) compared to similar results of other studies (columns A and B).

TABLE 6. Summary and comparison of properties of milks.

Lines A and B from Cox and Mueller (1937).

*From Davies, W. L. (1939).

| | Specific Gravity (°C) | % Protein | % Carbo-hydrate | % Total Solids | % Ash | pH |
|---|---|---|---|---|---|---|
| A. *Rattus* | 1.047 (25°) | 11.47 | 2.83 | 31.7 | 1.5 | 6.6 |
| B. *Bos* | 1.031 (25°) | 3.42 | 4.75 | 12.7* | 0.75 | 6.7 |
| C. *Bos* | 1.034 (22°) | 3.06 | 4.48 | 11.4 | 0.66 | - - |
| D. *L. nivalis* | 1.026 (22°) | 4.37 | 5.39 | 12.1 | 0.63 | 6.8 |
| E. *T. brasiliensis* | 1.034 (22°) | 11.07 | 3.70 | 34.4 | 0.73 | 6.3 |

## DISCUSSION

The pH and the specific gravity of the milks analyzed in this study indicated no significant differences. The specific gravity of the bovine controls compared favorably with known values and were only slightly higher than values for bat milk.

The results obtained from lactose determinations showed a significantly higher percent lactose for *L. nivalis*, a pollen and nectar feeder, than for *T. brasiliensis*, an insect feeding bat. The lactose determined for the bovine samples compare favorably with known values and show a significantly higher percent lactose than *T. brasiliensis*. A direct relationship is implicated here between the amount of dietary carbohydrate and the percent lactose found in the milk of these species. A closer examination of other species of bats would be necessary in order to establish such a dietary correlation but the implied relationship is interesting.

The colorimetric method and the difficulties inherent

in reading the percent lactose from a plotted standard curve may have contributed in part to the observed margin of error between trials.

T. brasiliensis milk showed a significantly higher percent total solids and percent protein than the same values obtained for L. nivalis or Bos. Whether or not this difference can be attributed to diet was not determinable in this study.

The high percent of total solids in T. brasiliensis milk may appear to contradict the results of the specific gravity determinations, but it has been pointed out by Jenness and Patton (1959) that a high protein content may be accompanied in milk by a correspondingly high fat content. Assuming this to be the situation, the one would tend to offset the other in specific gravity determinations of whole milk samples.

The high percent protein determined in T. brasiliensis milk confirms the value obtained for total solids in the milk of this species. As was previously mentioned, the percent protein values are a relative measure based upon the total nitrogen content of whole milk samples. It was

evident from observing the weight of the freeze-dried proteins that the greater percentage of this protein is casein although no determinations were specifically made for casein.

Casein is defined as the acid-precipitable fraction of whole milk and is a phospho-protein with ester-linked ionizable phosphate groupings. The rate of casein migration in an electrophoretic system has been correlated directly to the number of ionizable phosphate groups (Jenness and Patton, 1959).

The import of the work by Sloan and his group (1961) is the correlation made between milk protein patterns and the degree of 'primitiveness'[1] of origin for any order of mammals. These investigators designate a protein as 'primitive' because it is found in an animal whose immediate ancestors, based on morphological data, are believed to have appeared relatively early in mammalian history.

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[1] 'Primitive', as referred to by Sloan is interpreted to mean the degree of 'recentness' of origin of any living mammalian stock. Designating any one order of animals as primitive or advanced is purely hypothetical, and at best, can be applied to only one character in a single line of descent; or to the relative degree of specialization of any one character in animals at the same level.

The assumption that a protein has remained the same, or even relatively so, since the first appearnce of a line giving rise to any animal, is not necessarily valid simply because morphological characters have remained essentially unchanged.

In the light of what has been postulated for correlating the electrophoretic patterns of milk proteins with the phylogenetic origins of mammals one would expect the patterns of the milk proteins of Chiroptera to correspond to the "most primitive" patterns determined since they are, apparently, descendents of relatively early appearing mammals, the insectivores. The present study, however, shows that this is not the case in at least two species of bats in different families. The casein electrophoretic patterns of T. brasiliensis and L. nivalis do, in fact, represent Sloan's "most advanced" casein pattern, showing three peaks which greatly resemble the bovine casein electrophoretic peaks.

The electrophoretic whey protein patterns seem to be even less valid as phylogenetic criteria since they are influenced so profoundly by blood serum proteins and by the animal's lactation stage (Sloan et al., 1961).

The whey protein patterns of the two bats examined appear to be distinctly different, but it must be remembered that these are pooled samples from various undetermined lactation stages. It is of interest to note the electrophoretically positive component in the two bat whey protein patterns (i.e., the fraction moving to the left). This fraction has appeared in some perissodactyls and carnivores, and is possibly the basic enzyme lysozyme (Sloan et al., 1961).

When comparing the electrophoretic milk protein patterns, it is only possible to indicate general similarities within a given group of mammals. That similarities occur between groups (The Orders Artiodactyla, Perissodactyla, Carnivora, and Primates), or that differences occur among the same group (the Rodentia, Scivrus, Neotoma, and Cavia) has previously been pointed out (Sloan et al., 1961). The present study has added additional discrepancies, the sum of which indicates that further biochemical studies are both necessary and desirable in order to better define the phylogenetic significance of this character. In this regard the Order Chiroptera may present an ideal study group. They show a distinct group specialization, they are an old group phylogenetically and demonstrate considerable species radiation

into various ecological niches. This latter factor may be especially important when considering this group's nutritional diversity.

## SUMMARY AND CONCLUSIONS

Milk from two species of bats, *Leptonycteris nivalis*, Family Phyllostomatidae and *Tadarida brasiliensis*, Family Molossidae, known to utilize different types of food, was analyzed for the following chemical and physical properties: 1. Specific gravity. 2. pH. 3. Total solid and ash content. 4. Percent lactose. 5. Percent total nitrogen and protein. 6. Electrophoretic properties of casein and whey proteins. 7. Qualitative chromatographic determination of sugar.

Although several new facts were determined in this study, more questions have been posed than have been answered.

An analysis of the data presented above indicates that the two species of bats examined differ considerably in the composition of their milk: 1. *L. nivalis*, a pollen and nectar feeding bat, has a higher percentage of milk sugar than does *T. brasiliensis*, which feeds on insects (or than *Bos* which is a herbivore). 2. *T. brasiliensis*

on the other hand has a much greater protein content than does L. nivalis (or than Bos which is similar in this regard to L. nivalis). In order to relate these differences to diet, it would be necessary to examine milk of other bats which have similar diets and of those which have different diets.

The only sugar detected in paper chromatographic analysis was lactose, but the controls were not stringent enough to exclude all possibility of other sugars being present.

The physical properties measured in this study, pH and density, remained similar despite the wide variations in milk composition. From the density and percent protein determined for T. brasiliensis milk it is postulated that milk of this species would have a relatively high percent composition of fat.

The electrophoretic patterns of casein and whey protein provide a convenient analytical tool for some highly important milk components (important dietetically and perhaps phylogenetically). However, because of the complexities inherent in the analytical method and in phylogenetic concepts, the interpretation of results remains vague. To ascribe a

degree of "primitiveness" to any specific electrophoretic pattern can only be done in a most general and qualified way. As determined, the casein patterns of L. nivalis and T. brasiliensis show no obvious resemblance to those of marsupials or of "more primitive" patterns found in Mustela, Mephitis, Procyon, and Homo.

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