The absence of inhibitory effect of urethane (given in doses which reduce mitosis) on tissue metabolism indicates that urethane possibly inhibits some specific cell process or prevents the utilization of the energy of glycolysis or respiration from synthetic processes. Studies on the effect of methyl-di(2-chloroethyl)-amine (HN2) on the metabolism of the Walker carcinoma showed a decrease in glycolysis some days after treatment (Boyland, Clegg, Koller, Rhoden & Warwick, 1948). Urethane resembles nitrogen mustard in its cytological effects, and a similar effect on metabolism had been expected. The inhibition of glycolysis observed with nitrogen mustard is probably due to effects on hexokinase, and this may not be the fundamental cause of the nuclear damage, but rather an indication that phosphokinases are affected. Urethane may inhibit such phosphokinases (as yet unidentified) which are concerned with nucleoprotein metabolism, but the biochemical mechanism of its action is as yet unknown.

SUMMARY

1. As urethane is hydrolyzed by cold caustic alkali to give quantitative yields of ethanol, it can be

estimated by incubation with potassium hydroxide in Conway units and determination of the ethanol absorbed in acid potassium dichromate. This method can be applied directly to blood (using 0.2 ml.), urine or tissue extracts.

2. Following injection, urethane becomes evenly distributed in all the tissues of the body. Only a small amount is excreted in urine but the greater part is metabolized. The metabolism is slower in tumour-bearing rats than in normal rats.

3. Of two patients treated with urethane the blood of one showed a decrease in true cholinesterase while the blood pseudocholinesterase of the other patient decreased.

4. Treatment of rats or mice with urethane in doses sufficient to cause inhibition of mitosis or induce cancer of the lung did not reduce the respiration or glycolysis of lung, jejunum or Walker carcinoma tissue.

This investigation has been supported by generous grants to The Royal Cancer Hospital from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the U.S. Public Health Service.

REFERENCES

- Archer, H. E., Chapman, L., Rhoden, E. & Warren, F. L. (1948). *Biochem. J.* **42**, 58.
 Boyland, E., Clegg, J. W., Koller, P. C., Rhoden, E. & Warwick, O. H. (1948). *Brit. J. Cancer*, **2**, 17.
 Boyland, E. & Koller, P. C. (1949). (To be published.)
 Dustin, P. (1947). *Nature, Lond.*, **159**, 794.
 Fisher, K. C. & Henry, R. J. (1944). *J. gen. Physiol.* **27**, 469.
 Greenstein, J. P. (1943). *J. Nat. Cancer Inst.* **3**, 397.
 Greenstein, J. P., Jenrette, W. V., Mider, G. B. & White, J. (1941). *J. Nat. Cancer Inst.* **1**, 687.
 Hawkins, J. A. & Murphy, J. B. (1925). *J. exp. Med.* **42**, 609.
 Hawkins, R. D. & Gunter, J. M. (1946). *Biochem. J.* **40**, 192.
 Meyerhof, O. & Wilson, J. R. (1948). *Arch. Biochem.* **17**, 153.
 Nettleship, A. & Henshaw, P. S. (1943). *J. Nat. Cancer Inst.* **4**, 309.
 Paterson, E., ApThomas, I., Haddow, A. & Watkinson, J. M. (1946). *Lancet*, **i**, 677.
 Schmiedeberg, O. (1885). *Arch. exp. Path. Pharmacol.* **20**, 203.
 Skipper, H. E., Bryan, C. E., White, C. & Hutchison, O. S. (1948). *J. biol. Chem.* **173**, 371.
 Weil-Malherbe, H. & Schade, R. (1948). *Biochem. J.* **43**, 118.
 Westphal, U. (1943). *Hoppe-Seyl. Z.* **276**, 213.
 Winnick, I. (1942). *Industr. Engng Chem. (Anal. Ed.)*, **14**, 523.

An Immune Globulin Fraction from Bovine Precolostrum

By E. I. McDOUGALL,* *Institute of Animal Pathology, University of Cambridge*

(Received 20 August 1948)

This paper describes the preparation and partial characterization of an immune globulin fraction from bovine precolostrum. This material was studied as part of a joint programme on diseases of young calves because of its possible relation to colostrum, and the role of the latter in the transference of passive immunity to the suckling animal.

* Present address: Rowett Research Institute, Aberdeen, Scotland.

Bovine precolostrum is a viscous honey-like substance obtained from the udders of pregnant heifers at about half term. It was first described by Woodman & Hammond (1923) and Asdell (1925), who showed that it was deficient in the normal constituents of milk, but very rich in globulin. They suggested that it might prove rich in antibodies, a point recently demonstrated by Blakemore (1947), and that its accumulation in the udder well before

calving and its subsequent dilution by a true lacteal secretion would account for the composition and properties of colostrum and the rapid change from colostrum to milk after calving.

Colostrum itself was shown by Orcutt & Howe (1922) to be rich in easily salted-out globulins and antibodies, both of which were absent from the serum of the newborn calf, but appeared therein after the ingestion of colostrum. This work explained the experiments of Smith & Little (1922), which showed that feeding colostrum prevented losses of calves from infections in the first week of life, and suggested

that one of the most important constituents of colostrum was an immune globulin. Such a protein has recently been prepared from this source and investigated by Smith (1946*a, b*).

An immune globulin from bovine precolostrum has been investigated in the present work. This secretion has been examined for Howe's (1922) nitrogen fractions, bulked samples fractionated, the fractions examined for the distribution of antibody and the properties of an immune globulin fraction investigated, mainly by physico-chemical methods.

Table 1. *Nitrogen fractions in bovine precolostrum*

(Fractionation by a modification of Howe's (1922) method.)

Sample	Nitrogen (g./100 ml.)						
	Total	Globulin	Casein	Albumin + non-protein	Euglobulin	Pseudo-globulin I	Pseudo-globulin II
Heifer 7	4.72	3.61	0.62	0.49	1.10	2.12	0.39
Heifer 16	3.62	2.75	0.50	0.37	1.85	0.89	0.01
Bulk I	5.04	4.60	0.20	0.23	3.17	1.23	0.20
Bulk II (diluted)	2.23	2.05	0	0.18	1.31	0.70	0.04

Table 2. *Fractionation, method I*

EXPERIMENTAL AND RESULTS

Nitrogen fractions

It has been noted that bovine colostrum is rich in easily salted-out globulins, viz. the euglobulin and pseudoglobulin I fractions of Howe. Accordingly, to see how the proteins of precolostrum compared with those of colostrum in this respect, the distribution of nitrogen in different fractions was investigated in four samples of precolostrum using a modification of Howe's (1922) method.

Table 1 shows that of the globulin N, which forms the greater part of the total N, a high proportion belongs to the more easily salted-out euglobulin and pseudoglobulin I fractions. In this respect it resembles the globulin N of bovine colostrum. If the antibodies in the two secretions have similar salting-out limits, then precolostrum should be a good source of an easily salted-out immune globulin fraction.

Bulk fractionation

Two lots of bulked samples of precolostrum, bulk I and bulk II (diluted) of Table 1, were accumulated for fractionation. The methods of fractionation employed depended on differences of solubility in

Table 3. *Fractionation, method II*

The globulin was divided into euglobulin, pseudoglobulin I and pseudoglobulin II fractions by precipitation with 14, 18 and 22% (w/v) Na_2SO_4 at 34°. Casein, however, was estimated by acidification of the dilution in saline used for the total N estimation with acetic acid, and not by acidification of the filtrate from 14% (w/v) Na_2SO_4 , as in the original procedure; allowance was made for its precipitation by 18% (w/v) Na_2SO_4 in calculating pseudoglobulin I. This modification seemed justified as, on comparison of the two procedures in the analysis of precolostrum, the former gave lower values than the latter, and this suggested that proteins additional to casein were precipitated when the original method was used. Consequently, the present values for casein are slightly lower and for pseudoglobulin I slightly higher than would otherwise have been the case.

water and concentrated ammonium sulphate solutions respectively and are given schematically in Tables 2 and 3.

In both methods the bulked samples were first diluted in about 450 ml. saline, any fat and casein were removed by acidification, and the pH readjusted to approximately neutral. $(\text{NH}_4)_2\text{SO}_4$ was introduced by dialyzing in the calculated amount of salt from a rotating cellophan sac, and precipitates were removed by centrifugation.

In the first method the total globulins were separated at 2.05 M $(\text{NH}_4)_2\text{SO}_4$, and dialyzed against water to give fraction euglobulin A, the supernatant was adjusted to pH 5.5 and separated into euglobulin B, and pseudoglobulin fractions. The euglobulin fractions were finally dissolved in saline.

In the second method the solution of precolostrum was brought to 33% saturation with $(\text{NH}_4)_2\text{SO}_4$ to give a precipitate of fraction 0/33; the supernatant was brought to 40% saturation and the precipitate rejected; the filtrate from this was then brought to 50% saturation to give a precipitate of fraction 40/50: the filtrate from this was rejected. The two fractions were dissolved in saline to give a rather turbid white solution (0/33) and a clear pink-brown solution (40/50).

An approximate N balance sheet of both fractionation procedures was obtained by measuring the volume and N content of solutions at different stages of the procedure. The results are given in Table 4. In method I, the water-soluble pseudoglobulin fraction accounted for 72% of the globulin initially separated and in method II, the readily salted-out fraction 0/33 contained 74% of the total N in the starting material.

colostrum has been reported in two papers. Orcutt & Howe (1922) found an upper limit of 16.5 g./100 ml. for the precipitation of antibodies to *Escherichia coli* and *Brucella abortus* by sodium sulphate, and Smith (1946*a, b*) prepared an immune globulin fraction by precipitation at 0.3 saturation with ammonium sulphate. The present antibody globulin of precolostrum therefore resembles that of colostrum in its salting-out behaviour.

The 0/33 $(\text{NH}_4)_2\text{SO}_4$ fraction would be described as a euglobulin in Sørensen's terminology. However, it has been seen that the antibody was also found in the pseudoglobulin fraction obtained by dialysis. The terms euglobulin and pseudoglobulin therefore need some discussion. They have

Table 4. *Distribution of nitrogen and antibody in fractionation experiments*

Fraction	Approx. vol. (ml.)	(g./100 ml.)	N content of fraction			Titre to <i>Brucella</i> <i>abortus</i>
			Total in fraction (g.)	As % of globulin N	As % of total N	
Fractionation method I						
Dilute precolostrum	450	1.48	6.65	—	—	320
Dilute precolostrum less fat and casein	500	1.14	6.03	—	—	320
Total globulins	225	1.35	3.36	—	50.5	320
Euglobulin <i>A</i>	15	0.06	0.01	0.3	0.15	Absent
Pseudoglobulin + euglobulin <i>B</i>	200	1.20	3.00	—	—	320
Euglobulin <i>B</i>	100	0.40	0.57	17.0	8.6	Absent
Pseudoglobulin	175	0.97	2.42	72.0	36.9	320
Albumin, casein and losses	—	—	3.65	—	—	—
Fractionation method II						
Dilute precolostrum	450	2.23	10.0	—	—	—
Fraction 0/33	500	1.40	7.4	—	74	320
Fraction 40/50	150	0.16	0.25	—	2.5	Absent
Albumin, casein, fraction 33/40 and losses	—	—	2.35	—	23.5	—

Distribution of antibody

The agglutinin to *Brucella abortus* is one of the antibodies that have been studied in bovine colostrum. This is because it is a convenient antibody for many purposes since it occurs frequently in this species and can be easily titrated, and not because it is of any importance to the newborn calf. This antibody was known to have been present in the bulk samples of precolostrum used in the present work. Its distribution in the various fractions obtained above was therefore determined as a guide to the nature of the globulin of this and possibly of other antibodies in the secretion. As shown in Table 4, it was present in the pseudoglobulin fraction obtained by dialysis in method I and in fraction 0/33 obtained by salting out in method II and was absent from the remaining fractions.

The distribution of antibody in the ammonium sulphate fractions shows that the antibody globulin was a readily salted-out globulin. This property provides a broad basis for comparison with other proteins that are possibly related. The salting-out behaviour of the immune globulins of bovine

been applied to the water-insoluble and water-soluble fractions obtained by dialysis, to proteins precipitating in the ranges 0–33 and 33–50% saturation with $(\text{NH}_4)_2\text{SO}_4$, to proteins precipitating in the ranges 0–14 and 14–22% Na_2SO_4 respectively and to fractions obtained by precipitation at different concentrations of ethanol. It is possible to adjust the conditions of these precipitations to yield quantitatively similar results, but this in itself is insufficient proof that the fractions obtained by the different methods are qualitatively the same or are definite chemical entities. In fact, both fractions obtained by dialysis of serum proteins have been shown to be electrophoretically pauci-disperse, and salting-out methods have yielded fractions containing a single electrophoretic component which can be further separated into water-soluble and water-insoluble fractions by dialysis. It is evident from the present results that antibody globulin cannot be described as an euglobulin or a pseudoglobulin without qualifying the term by the conditions of separation.

Electrophoretic examination of fractions 0/33 and 40/50 in solution

As the relation between protein fractions obtained by dialysis and the original protein is uncertain, and as salting-out methods have been used successfully

in separating serum globulins, further examination of the fractions described in the preceding section was limited to those obtained by salting out with ammonium sulphate. These fractions were examined by electrophoresis in the Tiselius apparatus to obtain information on their purity and to enable some comparison to be made with other proteins.

The two fractions, were examined at 4° using a simple schlieren optical system, 1 month after preparation; and at

20° using the Philpot diagonal schlieren optical system, 7 months after storage at 4°. The velocity of each component was obtained either graphically by following its migration with a ground-glass scale or a cathetometer, or by measuring photographs taken near the start and finish of the electrophoresis, or by both methods.

The number and proportion of the chief components were similar at the two temperatures, but the mobilities were appreciably higher at 20° than at 4° (see Table 5 and Fig. 1). At 4°, fraction 0/33 showed a single strong band with a

Table 5. Electrophoretic examination of fractions 0/33 and 40/50 in the Tiselius apparatus

Temperature	...	...	...	4°						20°						
Fraction	...	...	...	...	0/33		40/50		...	0/33		40/50				
Buffer	...	...	...	Phosphate		Borate		Phosphate	Phosphate		Phosphate					
pH	...	...	...	7.0		8.5		7.0	7.5		7.5					
Ionic strength	...	...	...	0.1		0.06		0.1	0.1		0.1					
Protein concentration (g./100 mol)	...	...	...	1.0		1.0		1.0	1.5		1.0					
Mobility (μ./sec./V./cm.)																
Component	Asc.		Desc.		Asc.		Desc.		Asc.		Desc.		Asc.		Desc.	
1	—		—		—		—		—		—		1.61		1.37	
2	—		—		—		—		1.66		1.37		—		1.27	
3	—		—		—		—		1.02		0.93		—		1.03	
4	—		—		—		—		1.20		—		—		—	
5	—		—		—		—		1.02		0.84		—		—	
6	0.38		0.40		0.45		0.37		0.74		0.54		0.88		—	
7	0.20		0.16		0.23		0.24		0.41		0.36		0.65		0.50	
	—		—		—		—		—		—		—		—	
Chief component	6		5		4		4		6		5		4		—	
Coloured component	5		4		4		4		5		4		—		—

Fig. 1. Electrophoresis of protein fractions in phosphate buffer at pH 7.5 and 20°: (a) fraction 0/33, 1.5 g./100 ml., $E=4.3$ V./cm.; (b) fraction 40/50, 1 g./100 ml., $E=3.0$ V./cm. Nos. 1, 2, 4, 5, 6 in the diagram indicate components.

mobility of approximately $0.2 \mu/\text{sec.}/\text{V.}/\text{cm.}$ at pH 7.0 in phosphate buffer and at pH 8.5 in borate buffer; this band became rather diffuse at the end of a day's run: a second component with a mobility of $0.4 \mu/\text{sec.}/\text{V.}/\text{cm.}$ was detected by its brownish pink colour, but was not present in sufficient concentration to give a band. Fraction 40/50 showed six bands, some of them only just discernible: the chief component, no. 4, had a mobility of approximately $0.5 \mu/\text{sec.}/\text{V.}/\text{cm.}$ at pH 7.0 in phosphate buffer: the pink colour appeared to be present and to move at the same rate.

At 20° , fraction 0/33 showed one main peak with a mobility of approximately $0.4 \mu/\text{sec.}/\text{V.}/\text{cm.}$ at pH 7.5 in phosphate buffer, which became squat towards the end of the day's run. The coloured substance could be seen but gave no peak; it had an average mobility of $0.6 \mu/\text{sec.}/\text{V.}/\text{cm.}$ A number of small peaks were visible which were probably due to convectional disturbances. Fraction 40/50 again showed six components; the chief component, no. 4, had a mobility of approximately $0.9 \mu/\text{sec.}/\text{V.}/\text{cm.}$ at pH 7.5 in phosphate buffer.

The differences in mobilities in the two sets of experiments are greater than would be expected to arise from the small differences in pH of the measurements, as the latter were made on the alkaline side of the isoelectric point where the slope of the pH-mobility curve of proteins is least. They are probably due to the effect of temperature on viscosity, since an approximate correction for the latter, using the viscosity data for water of Svedberg & Pedersen (1940), reduces the mobilities of the chief components of fractions 0/33 and 40/50 at 20° from 0.38 and $0.93 \mu/\text{sec.}/\text{V.}/\text{cm.}$ to 0.24 and $0.59 \mu/\text{sec.}/\text{V.}/\text{cm.}$, at pH 7.5 in phosphate buffer, at 4° and these values are sufficiently close to the experimental results at 4° , at pH 7.0 in phosphate buffer, namely, 0.18 and $0.51 \mu/\text{sec.}/\text{V.}/\text{cm.}$ Unfortunately it was not possible to extend the present work to include measurements at a series of different pH values at the two temperatures and so describe the electrophoretic properties of the fractions more completely.

Comparison of the present results at the appropriate temperature can be made with those obtained by other workers on proteins of bovine origin: this is shown in Table 6, where the mobilities have been

rounded off to the first decimal place, and when necessary the average of the ascending and descending mobilities has been taken. The mobility of the chief component of fraction 0/33 at 4° does not correspond to that of any of the normal serum globulins, being about twice as fast as bovine γ -globulin: it is approximately the same as that found for the immune globulin of colostrum and whey, and the T-component of immune plasma by Smith (1946*a, b*), the component i_2 , of colostrum described by Gronwall (1947) and the γ -globulin appearing in the sera of young calves on the ingestion of colostrum, reported by Jameson, Alvarez-Tostado & Sortor (1942) and San Clemente & Huddleson (1943).

The mobility of the main component of fraction 40/50 at 4° was similar to that of the main component of fraction A, casein, of colostrum, described by Smith (1946*a, b*) and to that of component i_1 , one of the characteristic proteins of colostrum found by Gronwall (1947). As the amount of casein in precolostrum was very small and any present was removed in the fractionation process, the main component of this fraction is possibly the same as the protein i_1 , referred to above. The mobility of the fastest component of fraction 40/50 at 20° is similar to that found by Pedersen (1937) for lactoglobulin at this temperature.

The coloured component appearing in both fractions showed a mobility similar to that of other proteins described in colostrum, but as the colour of the latter has received no special comment, the resemblance is probably superficial.

Fraction 0/33, it has been noted, contained one main component. The diffuseness of the bands in the experiments at 4° , and the flatness of the curves in the experiments at 20° , appear greater than might be expected to arise from diffusion alone, and suggest that the preparation was heterogeneous. Unfortun-

Table 6. Comparison of mobility of precolostral proteins and of other bovine proteins

Reference	Protein	Mobility* ($\mu/\text{sec.}/\text{V.}/\text{cm.}$)	Temp. ($^\circ$)	pH	Buffer
Present paper	Precolostrum, fraction 0/33	0.2	4	7.0 8.5	Phosphate Borate
Smith (1946 <i>a, b</i>)	Colostrum, immune globulin	0.2	1	8.4	Veronal
Smith (1946 <i>a, b</i>)	Whey, immune globulin	0.2	1	8.6	Veronal
Smith (1946 <i>a, b</i>)	Plasma, T-component	0.2	1	8.6	Veronal
Smith (1946 <i>a, b</i>)	Plasma, γ -globulin	0.1	1	8.6	Veronal
Gronwall (1947)	Colostrum, component i_2	0.2	—	7.7	Phosphate
Jameson <i>et al.</i> (1942)	Serum of suckling calf, γ -globulin	0.2	0.5	7.7	Phosphate-saline
Present paper	Precolostrum fraction 40/50, chief component	0.5	4	7.0	Phosphate
Smith (1946 <i>a, b</i>)	Colostrum, fraction A, casein, chief component	0.5	1	8.4	Veronal
Gronwall (1947)	Colostrum, component, i_1	0.5	—	7.7	Phosphate
Present paper	Precolostrum, fraction 40/50, fastest component	1.5	20	7.5	Phosphate
Pedersen (1937)	Lactoglobulin	1.5	20	7.5	Phosphate

* These figures have been rounded off to the first decimal place.

ately, the runs had already taken too long for it to be practicable to test this point by reversal of the current. However, the heterogeneity constant (H), defined by Sharp, Hebb, Taylor & Beard (1942), was assessed from the second moments of the gradient curves of Fig. 1*a* and the values 0.047 and 0.054 μ ./sec./V./cm. obtained for the ascending and descending boundaries respectively; these values would be slightly reduced by allowing for the effect of diffusion on a slow-moving boundary: they compare with the value found by Sharp *et al.* (1942) for their horse pseudoglobulin I preparation, viz. 0.07 μ ./sec./V./cm. The heterogeneity was further tested by calculating the third and fourth moments of the curves of Fig. 1*a* at the end of the run: these indicated that the mobilities were normally distributed.

The heterogeneity found might have arisen during the course of fractionation which involved ammonium sulphate precipitation and dialysis at room temperature and extended over some 3 weeks, but this cannot be asserted without comparable data for this component before fractionation, which were not obtained in the present investigation.

Further examination of fraction 0/33

Of the two fractions obtained by salting out, fraction 0/33 was found to contain an antibody globulin and to consist of one electrophoretic component. The properties and purity of this fraction were therefore further investigated. The electrophoretic behaviour of the adsorbed protein was studied to give the iso-ionic point of the protein and to allow a comparison of the mobilities of the dissolved and adsorbed protein; the mean molecular weight was studied by osmotic pressure measurements; the number of thermodynamic components was investigated by solubility methods and the nitrogen content and the partial specific volume determined.

Electrophoresis of the adsorbed protein. The mobility of the protein adsorbed on droplets of Nujol (liquid paraffin) in buffers of varying pH and ionic strength was determined in a micro-electrophoresis cell in an ultramicroscope. The emulsion was made by running a boiling solution of 1 ml. Nujol in 20 ml. ethanol through a fine capillary tube into boiling water (1 l.), and removing the ethanol by boiling off about half the water. Acetate and phosphate buffers were made to cover eight pH values from 4.0 to 7.7, each at five different ionic strengths from 0.02 to 0.2, with the aid of a Cartesian nomogram based on the data of Green (1933). The solution for the mobility measurements was made by adding 1 ml. of 1.5 g./100 ml. solution of the protein in saline to 1 ml. of Nujol emulsion in a 50 ml. flask, adding the appropriate amounts of standard acetic acid and NaOH or KH_2PO_4 and KOH solutions, and making up to a volume with water. The solution after standing 15 min. was introduced into a double-tubed cell similar to that of Smith & Lisse (1936), but fitted with reversible Ag-AgCl electrodes in compartments separ-

ated from the rest of the cell by a constriction plugged with cotton wool. The electrodes were connected through a milliammeter and a reversing switch to a variable resistance connected across 200 V. d.c. mains. A voltmeter could be connected across the electrodes when desired. The applied potential was varied so that the current flowing through the cell did not exceed 5 ma., in order to avoid any convection disturbances through the heating effect of the current. The velocity of the adsorbed protein was obtained by timing ten droplets in both directions across a calibrated eye-piece micrometer, with a stopwatch reading to 0.1 sec. These measurements were made at the central stationary level of the smaller tube; this level was easily determined experimentally by a series of measurements at various apparent depths. The conductivity and pH of the solution were measured after its removal from the cell.

It was difficult to maintain constant conditions of measurement in some cases because of the heat produced by the lamp. Some typical figures in Table 7 show that the temperature

Table 7. *Constancy of conditions of measurement of mobility in the micro-electrophoresis cell*

		Potential difference (V.)					
Start	88	88	84	108	110	125	
Finish	88	86	84	110	110	127	
		Current (ma.)					
Start	4.3	4.28	4.0	3.97	4.2	4.5	
Finish	4.5	4.68	4.28	4.18	4.35	4.95	
		Temp. (°)					
Start	23	23	24	26	27.5	25	
Finish	24	25	25.5	27	28	25.5	

and (probably as a result of this) the current flowing increased, while the applied potential remained constant. Consequently, it was more convenient to calculate the potential gradient from the potential difference across, rather than from the current through, the cell. Any error involved in this procedure was small since good agreement was obtained with figures based on a mid value for the current, as shown in Table 8.

Table 8. *Comparison of the values of the potential gradient obtained from (a) the potential difference and the length of the capillary tube, and (b) the current and the conductivity of the solution*

Method of calculation	Potential gradient (V./cm.)							
	(a)	(a)	(a)	(a)	(a)	(a)	(a)	(a)
(a)	12.8	12.8	22.5	22.5	8.8	8.8	6.3	6.3
(b)	12.2	12.0	23.4	22.5	8.7	8.8	6.1	6.0

The final protein concentration obtained in this method, namely 0.03 g./100 ml., was shown to be sufficient to cover the droplets by (1) determining the pH mobility curve of Nujol droplets alone at 0.1 ionic strength, which showed mobilities several times greater than when protein was present; and (2) determining the mobility of droplets at pH 7.48 and ionic strength 0.1 in the presence of a decreasing concentration of protein; at the lowest protein concentration, 0.001 g./100 ml., this showed droplets of two distinct mobilities, indicating coated and uncoated droplets, and at higher protein

concentrations, a uniform value, decreasing slightly with increase of protein concentration, probably because of the effect of viscosity.

The pH-mobility curves were drawn from the data obtained at different ionic strengths (not included here). The appropriate mobility of the adsorbed protein, $0.97 \mu/\text{sec.}/V./\text{cm.}$, was considerably higher than that, $0.4 \mu/\text{sec.}/V./\text{cm.}$, found for the dissolved protein in the Tiselius apparatus at 20° , but was close to the value, $0.93 \mu/\text{sec.}/V./\text{cm.}$, found for the chief component of fraction 40/50 in solution.

The iso-ionic point of the adsorbed protein was derived from the pH-mobility curves as follows: (1) the mobility at different ionic strengths for each of eight constant pH values was interpolated and plotted against the square root of the ionic strength, giving lines (Fig. 2) that were extrapolated to

Fig. 2.

Fig. 3.

Fig. 4.

give the pH-mobility data at zero ionic strength (Fig. 3) from which an iso-ionic point of pH 5.13 was obtained; (2) the isoelectric points at the different ionic strengths were interpolated and plotted against the square root of the ionic strength (Fig. 4) and extrapolated to give an iso-ionic point of pH 5.15.

The different mobilities of the protein in the adsorbed and dissolved state may be explained by increased ionization on adsorption. There are, how-

ever, few proteins for which data are available for making a comparison like this. Horse-serum albumin, egg albumin and horse-serum pseudoglobulin were studied in solution by Tiselius (1930, 1937*a, b*) and adsorbed on inert particles by Abrahamson (1932) and Moyer (1938*a, b*). The data on serum albumin showed good agreement, but in the case of egg albumin, the pH-mobility curve of the adsorbed protein was slightly displaced to give an isoelectric point at pH 4.82 instead of at pH 4.55. The data for adsorbed pseudoglobulin showed good agreement with the early work of Tiselius in which the migration of the boundary was followed by ultraviolet adsorption at 20° . This agreement, however, requires qualification as Tiselius later resolved the pseudoglobulin into α - and γ -globulin with his improved apparatus working at 0° , so that this comparison has not been made on a pure protein. Adsorbed unfractionated horse-serum globulin was studied by Moyer & Moyer (1940), and was found to behave like the α -globulin of Tiselius, if allowance was made for the different temperatures of measurement; this suggested that adsorption had been selective. There is thus some evidence against the view that adsorption affects the ionization of a protein. This favours an alternative explanation of the present results, viz. that a trace of some substance more surface-active than the chief constituent of the fraction was present. This possibility is supported by similar mobilities noted for the adsorbed protein and the chief component of fraction 40/50 in solution.

Mean molecular weight. The osmotic pressure of a series of dilutions of the fraction in saline was determined in an Adair-type osmometer in a thermostat at 20° . The position of the descending inner meniscus was followed with a cathetometer; the initial rapid fall of the meniscus was succeeded by a slight but steady decrease due to slow denaturation of the protein. The equilibrium position was obtained by plotting the observations and extrapolating to zero time. At the end of the experiment, the osmometer was dismantled and the capillary correction, pH and nitrogen content of the solution determined. The corrected pressure p was assumed to be due to protein of concentration c , calculated from the nitrogen content and an experimentally determined factor 6.60 (see p. 540) as the Donnan effect of the protein in normal saline should be negligible.

The results are shown in the graph of p against c in Fig. 5. Except at the lowest concentrations, the points fall on a straight line which, however, does not pass through the origin. The molecular weight was evaluated by plotting p/c against c in three ways, shown in Fig. 6: (1) line *A* corresponds to the experimental data and indicates an uncorrected molecular weight of 170,000 at the higher concentrations, but is too curved to extrapolate to $c=0$; (2) line *B* is derived from the curve drawn to fit the experimental data and to pass through the origin; on extrapolation to $c=0$ it gives a molecular weight of 300,000; (3) line *C* is obtained from the experimental data adjusted to an origin at the intersection of the straight line through them with the c axis; on extrapolation to $c=0$ it gives a molecular weight of 146,000.

As the results obtained at the lowest concentrations are less accurate, because of the relatively greater capillary correction, than those obtained at higher concentrations, the latter, which fell approximately on a straight line, are more

Fig. 5.

Fig. 6.

significant and favour treatment (3) above. The failure of the line, however, to pass through the origin is difficult to interpret; it clearly cannot be due to any effect that is a function of the protein concentration. It may indicate that a constant amount of protein was denatured on setting up each osmometer or that the membranes had an asymmetry

potential giving anomalous osmosis. As there is no evident reason why either process should have occurred, it is probably more plausible to accept tentatively treatment (2) giving line *B* and an apparent molecular weight of 300,000.

This value is somewhat higher than those found for immune bovine colostrum globulins by Smith (1946*a, b*), 180,000, and for serum globulins by Adair & Robinson (1930), 175,000, and by Burk (1937), 173,000. It is quite different from that found for lactoglobulin by Pedersen (1937), 41,500 and 38,000 and for the immune globulin of bovine anti-pneumococcus serum by Kabat (1939), 910,000.

Solubility. As the purity of a preparation of high molecular weight is not established by any one criterion such as electrophoretic mobility, the number of thermodynamic components in the fraction was investigated by two solubility methods, namely, the salting-out curve of Cohn (1925)—solubility as a function of salt concentrations—and the phase test—solubility as a function of total amount of solute.

The salting-out curve. The protein solution (0.5 ml.) was added to 15 ml. portions of a series of dilutions of a 3*M*-phosphate buffer at pH 6.5, prepared according to Butler & Montgomery (1932), left standing in a water bath at 20° overnight, filtered through a no. 50 Whatman paper and the nitrogen determined on 2 ml. of the filtrate. The solubility is plotted on a logarithmic scale against the molarity of the phosphate buffer in Fig. 7. It is evident that the protein has been precipitated in two stages between the limits 1.2–1.4 and 1.4–2.1 *M*, the salting-out constants, K_s' , being 0.22 and 0.91 respectively.

Fig. 7.

The phase test. Increasing amounts of the protein solution were made up to 15 ml. with distilled water and phosphate buffer to give a final phosphate concentration of 1.4 *M* at pH 6.5; the solutions were then shaken vigorously and left standing in a water bath at 20° overnight, filtered through a no. 50 Whatman paper and the nitrogen determined on 2 ml. of the filtrate. The total N calculated from the volume and concentration of the protein solution used, and the soluble N found are given in Fig. 8. This shows three parts to the solubility curve, indicating saturation with components at *A* and *B* and the existence of at least a third component whose point of saturation was not attained. The

solubilities of the first two components were obtained from the intercepts of the extrapolated portions of the curve on the ordinate A' and B' and found to be, in terms of N, 0.06 and 0.27 mg./ml. respectively. The proportions determined graphically by the procedure shown in Fig. 9 were found to be 53 and 17% of the protein respectively.

is given in Table 9, which shows an average value of 15.15%, equivalent to a factor of 6.60. This value is a little lower than that reported by Smith (1946 *a, b*) for the immune globulin of bovine colostrum, namely 15.44 and 15.53%, but is close to the value of 15.2% found for serum globulin by Adair & Robinson (1930). The partial specific volume was found to be

Fig. 8.

It is clear from both these tests that the fraction contained several components, although electrophoresis had revealed only one. Comparison of the salting-out curve with the findings of Butler & Montgomery (1932) that the euglobulin and pseudoglobulin of human and of horse serum precipitated

0.723, a value close to the average of 0.75 for serum globulin, which suggests that the present fraction, like the γ -globulin of serum, is not associated with any appreciable quantity of lipids.

Table 9. Nitrogen content of protein fraction 0/33

N (g./100 ml. solution)	N (% of ash-free protein)
0.473	15.12
0.815	15.30
1.34	15.13
1.34	15.11
1.34	15.13
1.34	15.09
Average	15.15
N factor (=100/15.15)	6.60

DISCUSSION

The extent to which an immune globulin fraction can be regarded as a distinct entity is difficult to decide. Biological criteria show that many different antibodies may be chemically indistinguishable, and that the same antibody may have different chemical properties, depending on the species in which it is formed, the manner in which its formation is stimulated and the stage in the response to the antigenic stimulus. In the present work, tests were made for the *Brucella* agglutinin only, and this was found to be confined to fraction 0/33 of the salting-out fractions. As other antibodies of the secretion may have similar solubilities, this fraction is probably not biologically pure and may be described tentatively as the immune globulin of bovine precolostrum. The extent to which this fraction is chemically homogeneous may be judged from the present results in

Fig. 9.

between the limits 1.2–1.6M and 1.5–2.5M, and the finding of Butler, Blatt & Southgate (1935) that horse pseudoglobulin was salted out with $K'_s = 0.5$ in the same buffer system, suggests that the proteins in the present fraction are probably distinct from the main serum globulins.

Nitrogen content and partial specific volume. The N content and partial specific volume were determined on saline solutions of the fraction by drying to constant weight at 110°, and by pycnometry at 20° respectively. The N content

several ways. Electrophoresis of the dissolved protein showed one component probably accounting for all the fraction. Electrophoresis of the adsorbed fraction suggested that a minor component may have been preferentially adsorbed. The salting-out curve showed a chief component and one other, and the phase test a chief component and two others. In considering these results it should be borne in mind that electrophoresis of the adsorbed protein may afford a very sensitive qualitative test of the purity of the protein, as it would be affected by the presence of quite small amounts of other proteins if these were more surface active than the main constituent, and that the phase test is one of the most sensitive tests of purity available, since a protein may appear homogeneous in the ultracentrifuge and Tiselius apparatus and still fail this test. It is therefore considered that while the present fraction is not pure, it does represent a promisingly high degree of separation of the main component of precolostrum with which the immune properties of the latter appear to be associated.

The properties of an immune globulin from bovine precolostrum are not without significance in connexion with the formation of this material and its relation to the subsequent secretions from the bovine udder. This aspect of the present results and of other experiments will be discussed elsewhere by Blakemore & McDougall (unpublished). Here it may be pointed out that the resemblance noted between the main electrophoretic component of precolostrum and the T-component of bovine plasma suggests that the precolostrum globulins are secreted from the blood stream, a view which is supported by the antibody studies of Blakemore (1947). The globulins of precolostrum resemble those of colostrum, in that a high proportion of them are easily salted out; that the easily salted-out globulins bear the *Brucella* agglu-

tinins and probably other antibodies of the secretion; and that the main globulin fraction resembles the immune globulin of colostrum, in the mobility of its main electrophoretic component, its approximate molecular weight, and its salting-out limits. These similarities support the view on the interrelation of precolostrum, colostrum and milk mentioned in the introduction, and suggest that an adequate dry period is necessary before calving to yield the best colostrum.

SUMMARY

1. Bovine precolostrum globulins contain a high proportion of Howe's (1922) euglobulin and pseudo-globulin I fractions, i.e. those most easily salted out.

2. An easily salted-out immune globulin fraction has been prepared by ammonium sulphate precipitation at 33% saturation.

3. This fraction contained one electrophoretic component with a mobility of $0.2\mu./\text{sec.}/V./\text{cm.}$ at 4° , in phosphate buffer at pH 7.0 and ionic strength 0.1; but solubility tests in phosphate buffers indicated more than one component. It had an apparent molecular weight (tentatively proposed) of 300,000, a nitrogen content of 15.15% and a partial specific volume of 0.723.

4. The properties of this fraction are discussed in relation to the T-component of bovine plasma and the immune globulin of bovine colostrum.

I am indebted to Mr F. Blakemore, Veterinary Investigation Officer, Institute of Animal Pathology, Cambridge, for the supply of material and for the examination of the fractions for antibody; to Prof. E. K. Rideal and Dr P. Johnson, Colloid Science Department, Cambridge, for facilities for and encouragement in much of the physico-chemical work in the present investigations, and to Dr E. C. Bate-Smith, Superintendent, Low Temperature Research Station, Cambridge, for facilities for some of the electrophoresis measurements.

REFERENCES

- Abrahamson, H. A. (1932). *J. gen. Physiol.* **15**, 575.
 Adair, G. S. & Robinson, M. E. (1930). *Biochem. J.* **24**, 1864.
 Asdell, S. A. (1925). *J. agric. Sci.* **15**, 358.
 Blakemore, F. (1947). *Proc. 4th. Int. Congr. Microbiol.* Copenhagen. In the Press.
 Burk, N. F. (1937). *J. biol. Chem.* **121**, 373.
 Butler, A. M., Blatt, H. & Southgate, H. (1935). *J. biol. Chem.* **109**, 758.
 Butler, A. M. & Montgomery, H. (1932). *J. biol. Chem.* **99**, 173.
 Cohn, E. J. (1925). *Physiol. Rev.* **5**, 349.
 Green, A. A. (1933). *J. Amer. chem. Soc.* **55**, 2331.
 Gronwall, A. (1947). *Nature, Lond.*, **159**, 376.
 Howe, P. E. (1922). *J. biol. Chem.* **52**, 51.
 Jameson, E., Alvarez-Tostado, C. & Sortor, H. H. (1942). *Proc. Soc. exp. Biol., N.Y.*, **51**, 163.
 Kabat, E. A. (1939). *J. exp. Med.* **69**, 103.
 Moyer, L. S. (1938a). *J. phys. Chem.* **42**, 71.
 Moyer, L. S. (1938b). *J. biol. Chem.* **122**, 641.
 Moyer, L. S. & Moyer, E. Z. (1940). *J. biol. Chem.* **132**, 373.
 Orcutt, M. L. & Howe, P. E. (1922). *J. exp. Med.* **36**, 291.
 Pedersen, K. O. (1937). *Biochem. J.* **30**, 961.
 San Clemente, C. L. & Huddleson, I. F. (1943). *Bull. Mich. agric. Exp. Sta.* **182**, 3.
 Sharp, D. G., Hebb, M. H., Taylor, A. R. & Beard, J. W. (1942). *J. biol. Chem.* **142**, 217.
 Smith, E. L. (1946a). *J. biol. Chem.* **164**, 345.
 Smith, E. L. (1946b). *J. biol. Chem.* **165**, 665.
 Smith, M. E. & Lisse, M. W. (1936). *J. phys. Chem.* **40**, 399.
 Smith, T. & Little, R. B. (1922). *J. exp. Med.* **36**, 181.
 Svedberg, T. & Pedersen, K. O. (1940). *The Ultracentrifuge*. Oxford: Clarendon Press.
 Tiselius, A. (1930). *Nova Acta Soc. sci. upsal.* **7**, (4). (Quoted by Abrahamson, H. A., Moyer, L. S. & Gorin, M. H. (1942). In *Electrophoresis of Proteins*, ch. 4, p. 82. New York: Rheinhold.
 Tiselius, A. (1937a). *Biochem. J.* **31**, 313.
 Tiselius, A. (1937b). *Biochem. J.* **31**, 1464.
 Woodman, H. E. & Hammond, J. (1923). *J. agric. Sci.* **13**, 180.