Adenosine-Triphosphatase Activity and Nicotinamide Nucleotide Coenzymes in the Parotid Gland of the Young Lamb and Adult Sheep

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1. The activity of a Mg^{2+} -dependent Na^{+} -plus- K^{+} -activated adenosine triphosphatase and the concentrations of nicotinamide nucleotide coenzymes have been measured in the immature parotid glands of young lambs and in the actively secreting glands of adult sheep. 2. The activity of the adenosine triphosphatase increased during development and attained relatively high levels in the mature secreting gland. 3. A high $([NAD] + [NADH_2])/([NADP] + [NADPH_2])$ ratio (approx. 10:1) was observed in the parotid glands of lambs and sheep. 4. The high concentrations of NAD and the very low concentrations of $NADPH_2$ have been discussed in relation to metabolic activity, the activity of the Na^{+} -plus- K^{+} -activated adenosine triphosphatase and the secretion of saliva by the parotid gland.

The parotid gland of an adult sheep secretes 1–4 l. of alkaline hyperosmotic saliva daily. In the young lamb, however, the parotid gland is not functional during the first few weeks of life and only a very limited flow of saliva occurs. As the lamb becomes older, and the rumen develops to accommodate the large quantities of fodder that are ingested, the parotid glands also develop from the small immature glands characteristic of lambs less than 5 weeks old. The histological appearance of the gland changes and the functional activity characteristic of adult sheep can be observed (Wilson & Tribe, 1961). Kay (1958, 1960) has observed a similar development in the parotid glands and salivary secretion in young goats and calves.

In the secreting gland it is probable that the movement of ions is achieved by active transport systems (Burgen & Emmelin, 1961) that, in the parotids of adult sheep, would have to account for the secretion of a saliva containing about 180 m-equiv. of Na^{+} and 2 m-equiv. of K^{+} /l. (Denton, 1957).

Evidence now indicates that a Na^{+} -plus- K^{+} -activated adenosine triphosphatase is involved in cation transport across cell membranes (Skou, 1957; Blond & Whittam, 1964; Judah & Ahmed, 1964), and Schwartz, Laseter & Krintz (1963) have reported an enzymic basis for active cation transport in the parotid gland of the dog.

In view of the immaturity of the parotid gland in young lambs and the rapid development within a few weeks to the full functional activity characteristic of adult sheep, the activity of a Na^{+} -plus- K^{+} -

activated adenosine triphosphatase in the parotid gland of lambs and sheep was studied.

As the relative concentrations of oxidized and reduced NAD and NADP have been considered to reflect the metabolic state of various tissues (Dickens, 1961; Lowenstein, 1961; Caiger, Morton, Filsell & Jarrett, 1962), the concentrations of the oxidized and reduced forms of these coenzymes were also determined and assessed in relation to adenosine-triphosphatase activity.

EXPERIMENTAL

Animals. Merino sheep (five ewes aged 4 years, five ewe-lambs aged 6–8 weeks and six ewe-lambs aged 2–3 weeks) were used in these experiments. The adult sheep were grazing on an improved mixed pasture, and the lambs, born during May–June, were running with their dams on the same type of pasture. The animals were removed from the source of their food about 2 hr. before slaughter.

Procedure. The animals were killed by severing the neck and cervical cord, and the parotid glands were removed within 30 sec. and dropped into liquid N_2 . About 10 min. later the glands were ground to a fine powder in a stainless-steel pestle and mortar cooled in liquid N_2 . Weighed portions of the mixed frozen powder were taken for determinations of adenosine-triphosphatase activity, nicotinamide nucleotide coenzymes and DNA phosphorus.

On several occasions when the adenosine-triphosphatase activity of a sample of fresh unfrozen gland was determined the activity was approximately the same as that of a sample of the gland immersed in liquid N_2 . However, a more satisfactory homogenate was obtained when the finely-ground frozen powder was used.

Extraction and determination of adenosine-triphosphatase activity. Approx. 1 g. of tissue was weighed and homogenized

at 2000 rev./min. for 2 min. in 10 ml. of ice-cold sucrose buffer in a glass Potter-Elvehjem homogenizer with a Teflon pestle (A. H. Thomas and Co., Baltimore, Md., U.S.A.). The buffer had the following composition: 0.25 M-sucrose-0.2 mM-EDTA-0.2 mM-L-histidine hydrochloride, adjusted to pH 6.2 with tris. The homogenate was filtered through several thicknesses of cotton gauze and kept cold throughout the procedure.

Enzymic activity was determined by using a standard test system based on that described for guinea-pig kidney by Charnock & Post (1963). A series of preliminary experiments were carried out to determine the concentrations of Na^+ and K^+ , the pH of the system, the time of incubation and the amount of tissue that gave optimum activity for ruminant parotid gland.

The reaction mixture contained glycylglycine (100 mM), MgSO_4 (2 mM), EDTA (0.2 mM), ATP (2 mM), NaCl (80 mM), KCl (20 mM) (5 mM- Na^+ was contributed by the various reagents other than NaCl), and when present ouabain (0.1 mM), in a total volume of 2.5 ml. The reaction was carried out in 10 ml. Erlenmeyer flasks by shaking in a water bath for 30 min. at 37° and was started by adding either 0.03 or 0.05 ml. of homogenate after a preincubation period of 5 min.

The glycylglycine- MgSO_4 -EDTA mixture was made up as a stock solution and buffered to pH 7.6 with tris. The ATP solution and the NaCl-KCl mixture were also adjusted to pH 7.6 with tris. The appropriate amount of the stock solution was placed in each flask and the ATP, NaCl, KCl and ouabain were added before placing the flasks in the water bath.

The reaction was stopped by adding 1.5 ml. of ice-cold 8% (w/v) HClO_4 . The precipitated protein was removed by filtration (Whatman no. 42 paper). All flasks were kept in ice-water or in a cold room when not being incubated. The orthophosphate in the protein-free filtrate was measured by the method of Allen (1940). Adenosine-triphosphatase activity was expressed as m-moles of orthophosphate released/g. fresh wt./hr.

Extraction and determination of DNA phosphorus. The extraction procedure of Schneider (1945) and the method of Burton (1956) were used, with modifications as given by Jarrett & Filsell (1964).

Extraction and determination of nicotinamide nucleotide coenzymes. Approx. 1 g. of ground frozen powder was weighed and homogenized in ice-cold 0.5 M-trichloroacetic acid for determination of oxidized coenzymes or in hot 0.1 M- Na_2CO_3 for determination of reduced coenzymes. The details of extraction and assay of coenzymes have been reported by Caiger *et al.* (1962).

Chemicals. ATP (disodium salt) and ouabain (strophanthin-G) were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. The glycylglycine was laboratory-reagent grade and the EDTA (disodium salt), MgSO_4 , NaCl and KCl were AnalaR grade from British Drug Houses Ltd., Poole, Dorset. Chemicals used in the determination of the nicotinamide nucleotide coenzymes and the DNA phosphorus were the same as those listed by Caiger *et al.* (1962) and Jarrett & Filsell (1964).

RESULTS

In the system studied, the release of orthophosphate from ATP in the presence of Mg^{2+} , Na^+ and K^+ has been interpreted as the total adenosine-

Fig. 1. Effect of Na^+ , K^+ and ouabain on adenosine-triphosphatase activity (expressed as m-mole of orthophosphate liberated/g. fresh wt. of parotid tissue/30 min.). Samples (0.05 ml.) of homogenate in 2.5 ml. of medium containing the concentrations of Na^+ plus K^+ indicated were incubated at 37° for 30 min. ○, Total activity; ●, ouabain-inhibited activity; △, Na^+ -plus- K^+ -activated activity.

triphosphatase activity of the tissue (○ in Fig. 1). The presence of ouabain inhibits the Na^+ -plus- K^+ -activated adenosine triphosphatase (● in Fig. 1) and the activity then approximates to that found when either Na^+ or K^+ is absent from the medium. The decrease in activity due to ouabain is thus a measure of the Na^+ -plus- K^+ -activated adenosine triphosphatase (△ in Fig. 1).

In a series of determinations made with a constant amount of Na^+ (85 mM) maximum activity of the Na^+ -plus- K^+ -activated adenosine triphosphatase was obtained with K^+ concentrations between 20 and 160 mM. With a constant amount of K^+ (20 mM) maximum activity was obtained with Na^+ concentrations between 45 and 165 mM. In the standard system final concentrations of 85 mM- Na^+ and 20 mM- K^+ were used.

Determinations were made with mixtures at various pH values obtained by adding either hydrochloric acid or tris to the glycylglycine buffer. No significant pH change occurred during incubation. Optimum activity was observed at about pH 7.6 for both the total and the ouabain-inhibited adenosine triphosphatase. The Na^+ -plus- K^+ -activated adenosine-triphosphatase activity was maximal between pH 7.0 and 7.6 (Fig. 2).

In the standard test system the activity of the Na^+ -plus- K^+ -activated adenosine triphosphatase in various amounts of homogenate up to 0.06 ml. gave a linear response and the activity of the enzyme was also linear with time over a period of 35 min. incubation.

Table 1 shows the details of the animals used, together with the percentage dry weight of the parotid glands, the concentration of DNA phosphorus and an estimate of mean salivary flow obtained from the literature (Wilson & Tribe, 1961; Denton, 1957).

Table 2 shows the rate of orthophosphate liberation with and without ouabain, and the decrease due to ouabain, which represents the activity of Na⁺-plus-K⁺-activated adenosine triphosphatase in the parotid glands of lambs and sheep. The activity is

shown both as m-moles/g. fresh wt./hr. and as m-moles/mg. of DNA phosphorus/hr.

An analysis of variance of the data showed that there was a significant difference ($P < 0.01$) for the Na⁺-plus-K⁺-activated adenosine triphosphatase when expressed on a fresh-weight basis between the 2-3-week-old lambs (mean 0.20 m-mole/g./hr.) and the adults (mean 0.38 m-mole/g./hr.), and between the 2-3-week-old lambs and the 6-8-week-old lambs (mean 0.34 m-mole/g./hr.). As the percentage dry weight of the parotid glands did not change significantly between the various groups, the same relationship was observed when the adenosine-triphosphatase activity was expressed on a dry-weight basis. When assessed on the basis of activity/unit of DNA phosphorus, however, the difference between the 2-3-week-old lambs (mean 0.40 m-mole/mg. of DNA phosphorus/hr.) and the adults (mean 1.10 m-moles/mg. of DNA phosphorus/hr.) showed a probability of $P < 0.001$.

A highly significant difference ($P < 0.001$) was observed for the total adenosine-triphosphatase activity of the system when expressed on both a fresh-weight and DNA-phosphorus basis between the 2-3-week-old lambs (mean 1.7 m-moles/mg. of DNA phosphorus/hr.) and the adults (mean 4.9 m-moles/mg. of DNA phosphorus/hr.), and between the 2-3-week-old lambs and the 6-8-week-old lambs (mean 3.5 m-moles/mg. of DNA phosphorus/hr.). A difference was also observed between the 6-8-week-old lambs and the adults ($P < 0.01$) for this adenosine triphosphatase, although the Na⁺-plus-K⁺-activated enzyme showed no significant difference between these two groups.

Fig. 2. Effect of pH on adenosine-triphosphatase activity (expressed as m-moles of orthophosphate liberated/g. fresh wt. of parotid tissue/30 min.). Samples (0.05 ml.) of homogenate in 2.5 ml. of medium at the pH values indicated were incubated at 37° for 30 min. ○, Total activity; ●, ouabain-inhibited activity; △, Na⁺-plus-K⁺-activated activity.

Table 1. Age, body weight, percentage dry weight and DNA phosphorus in parotid glands of lambs and sheep

Animals		Body wt. (kg.)	Dry wt. (%)	DNA phosphorus (μg./g. fresh wt.)	Estimated mean salivary flow (g./min.)
Lambs (2-3 weeks old)	1	8.0	—	500	0.8
	2	6.7	25.6	527	
	3	7.8	28.4	491	
	4	6.9	23.0	568	
	5	7.7	29.0	416	
	6	10.0	27.6	545	
Lambs (6-8 weeks old)	7	15.2	26.6	442	5.0
	8	12.7	21.7	432	
	9	13.1	25.9	358	
	10	18.0	23.1	290	
	11	14.6	22.8	375	
Adult sheep	12	43.0	22.6	388	10.0
	13	42.5	24.1	340	
	14	44.2	21.2	350	
	15	45.5	23.6	315	
	16	42.7	26.1	296	

Table 2. *Adenosine-triphosphatase activity in parotid glands of lambs and sheep*

Samples of homogenate (0.03 or 0.05 ml.) were incubated at 37° for 30 min. in 2.5 ml. of medium containing (final concentrations) glycylglycine (100 mM), MgSO₄ (2 mM), EDTA (0.2 mM), ATP (2 mM), Na⁺ (85 mM), K⁺ (20 mM) and with or without ouabain (0.1 mM). The rate of orthophosphate liberated from ATP was a measure of the adenosine-triphosphatase activity. The values in columns headed 'Control' represent the total adenosine-triphosphatase activity of the system, and those in columns headed 'Decrease due to ouabain' represent the Na⁺-plus-K⁺-activated adenosine-triphosphatase activity.

Animals		Rate of orthophosphate liberation					
		(m-moles/g. fresh wt./hr.)			(m-moles/mg. of DNA phosphorus/hr.)		
		Control	With ouabain	Decrease due to ouabain	Control	With ouabain	Decrease due to ouabain
Lambs (2-3 weeks old)	1	0.69	0.51	0.18	1.38	1.02	0.36
	2	1.04	0.74	0.30	1.97	1.40	0.57
	3	0.78	0.67	0.11	1.59	1.37	0.22
	4	0.84	0.65	0.19	1.48	1.15	0.33
	5	0.89	0.65	0.24	2.14	1.56	0.58
	6	0.95	0.75	0.20	1.74	1.37	0.37
Lambs (6-8 weeks old)	7	1.30	1.05	0.25	2.94	1.37	0.57
	8	1.38	1.03	0.35	3.19	2.38	0.81
	9	1.33	1.03	0.30	3.72	2.88	0.84
	10	1.34	0.89	0.45	4.62	3.07	1.55
	11	1.19	0.86	0.33	3.17	2.29	0.88
Adult sheep	12	1.73	1.17	0.56	4.46	3.02	1.44
	13	1.66	1.35	0.31	4.88	3.97	0.91
	14	1.66	1.23	0.43	4.74	3.51	1.23
	15	1.56	1.25	0.31	4.95	3.97	0.98
	16	1.61	1.33	0.28	5.44	4.49	0.95

Table 3. *Nicotinamide nucleotide coenzymes in parotid glands of lambs and sheep*

Animals		Concn. of nicotinamide nucleotide coenzymes							
		(mμmoles/g. fresh wt.)				(mμmoles/mg. of DNA phosphorus)			
		NAD	NADH ₂	NADP	NADPH ₂	NAD	NADH ₂	NADP	NADPH ₂
Lambs (2-3 weeks old)	1	301	34	14	< 10	602	68	28	< 10
	2	339	52	11	24	643	99	21	46
	3	309	33	13	28	629	67	26	57
	4	321	48	10	29	565	85	18	51
	5	305	48	< 10	22	733	115	< 10	53
	6	306	39	16	34	561	72	29	62
Lambs (6-8 weeks old)	7	443	91	< 10	39	1000	206	< 10	88
	8	375	60	< 10	37	868	139	< 10	86
	9	573	85	< 10	50	1601	237	< 10	140
	10	503	63	11	30	1734	217	31	103
	11	445	73	20	31	1187	195	53	83
Adult sheep	12	630	90	< 10	53	1624	232	< 10	137
	13	542	71	10	54	1594	209	29	159
	14	576	97	47	36	1646	277	134	103
	15	570	82	10	48	1810	260	29	152
	16	537	101	18	36	1814	341	61	122

In Table 3 the concentrations of NAD, NADH₂, NADP and NADPH₂ in the parotid glands of lambs and sheep are expressed as mμmoles/g. fresh wt. and mμmoles/mg. of DNA phosphorus.

An analysis of variance of the data showed a

highly significant difference ($P < 0.001$) for NAD content (on the basis of both fresh weight and DNA phosphorus) between the 2-3-week-old lambs (mean 622 mμmoles/mg. of DNA phosphorus) and both the 6-8-week-old lambs (mean 1278 mμmoles/

mg. of DNA phosphorus) and the adults (mean 1697 μ moles/mg. of DNA phosphorus). A significant difference ($P < 0.01$) was observed between the 6–8-week-old lambs and the adults. The difference in the concentration of NADH₂ between the 2–3-week-old lambs (mean 84 μ moles/mg. of DNA phosphorus) and both the 6–8-week-old lambs (mean 199 μ moles/mg. of DNA phosphorus) and the adults (mean 264 μ moles/mg. of DNA phosphorus) was also highly significant ($P < 0.001$), but little difference was observed between the 6–8-week-old lambs and the adults. The concentration of NADP was not significantly different in any of the groups, and the concentration of NADPH₂ differed significantly ($P < 0.01$ on a fresh-weight and $P < 0.001$ on a DNA-phosphorus basis) between the 2–3-week-old lambs (mean 47 μ moles/mg. of DNA phosphorus) and the adults (mean 135 μ moles/mg. of DNA phosphorus).

DISCUSSION

Various workers have shown that metabolic differences exist between lambs and sheep, particularly in relation to carbohydrate and fatty acid metabolism (Reid, 1950; Jarrett & Potter, 1952; Jarrett & Filsell, 1964). Some metabolic changes that take place during the growth of the lamb can be directly associated with the development of the rumen and the increasing dependence of the animal on a large supply of lower fatty acids absorbed from this part of the alimentary tract. To enable an efficient degradation of the fodder by the microbial flora in the rumen, an adequate supply of saliva is necessary.

Wilson & Tribe (1961) have shown that, before 4 weeks of age, the parotid gland of a lamb is physiologically immature, secreting only 0.1–0.2 g. of saliva/g. of parotid tissue/min. The parotid gland develops rapidly from 4 to 6 weeks of age in a lamb running on pasture and soon reaches the adult capacity of about 0.6 g. of saliva/g. of tissue/min. The rate of flow of parotid saliva was shown to be significantly correlated with the fresh weight of rumen tissue.

Although no measure of salivary flow was determined in the present study a histological examination of the parotids from lambs and sheep in each age group showed the same degree of immaturity in the 2–3-week-old lambs, with differentiation in the 6–8-week-old lambs towards the adult structure, as described by Wilson & Tribe (1961).

With the development of the gland and the secretion of increasingly larger volumes of fluid containing a high concentration of Na⁺, it is likely that an active transport system is developed for the movement of ions into saliva.

It is now apparent that a Mg²⁺-dependent Na⁺-plus-K⁺-activated adenosine triphosphatase is associated with active cation-transport mechanisms, and this enzyme has been intensively studied in relation to the supply and utilization of energy for Na⁺ transport (see reviews by Hokin & Hokin, 1963; Judah & Ahmed, 1964).

Bonting, Simon & Hawkins (1961) compared the total and the Na⁺-plus-K⁺-activated adenosine-triphosphatase activity in a number of different tissues from the cat. The highest activity noted was in nervous tissue and in tissues concerned with secretory function. Bonting, Caravaggio, Canady & Hawkins (1964) have observed a very active Na⁺-plus-K⁺-activated adenosine triphosphatase in the salt gland of herring gulls. After gulls had been maintained for 7 weeks on a decreased Na⁺ load the Na⁺-plus-K⁺-activated adenosine-triphosphatase activity was decreased to about 50% of the original value.

Little work has been reported on the adenosine-triphosphatase activity in immature tissue, or in tissue that is undergoing rapid development towards an active physiological state. However, Samson, Dick & Balfour (1964) have investigated the activity of a Mg²⁺-dependent Na⁺-plus-K⁺-activated adenosine triphosphatase in the brains of rats during immediate post-natal development. Stimulation by Na⁺ and K⁺ was absent at birth, and increased rapidly during development over the next 10–20 days.

The present study on the parotid gland of the lamb has demonstrated a Na⁺-plus-K⁺-activated adenosine-triphosphatase system that increases in activity during development of the gland. Associated with this development is a marked increase in salivary flow. Whether the changes in the adenosine-triphosphatase activity are directly related to the increased rate of salivary flow or to some other changes associated with the maturing gland cannot yet be determined.

The results also show a concomitant change in the concentrations of nicotinamide nucleotide coenzymes. Caiger *et al.* (1962) and Filsell, Jarrett, Atkinson, Caiger & Morton (1963) have discussed the importance of the relative concentrations of oxidized and reduced nicotinamide nucleotide coenzymes in relation to metabolic changes in the livers of lambs and sheep. It was considered that a high $([NAD] + [NADH_2])/([NADP] + [NADPH_2])$ ratio could reflect an emphasis on oxidative metabolism.

In the present study a significant rise in the concentrations of NAD + NADH₂ and of NADP + NADPH₂ coincided with increased secretory activity, but the $([NAD] + [NADH_2])/([NADP] + [NADPH_2])$ ratio (approx. 10:1) remained unchanged during development. As the

concentration of $\text{NADP} + \text{NADPH}_2$ was very low compared with that in other tissues (Caiger *et al.* 1962), it is suggested that in the parotid gland of lambs and sheep there is little reductive synthetic activity. The relatively high concentration of $\text{NAD} + \text{NADH}_2$ may indicate an emphasis on oxidative pathways that lead to production of ATP as the source of energy for cation secretion in the parotid gland.

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REFERENCES

- Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
 Blond, D. M. & Whittam, R. (1964). *Biochem. J.* **92**, 158.
 Bonting, S. L., Caravaggio, L. L., Canady, M. L. & Hawkins, N. M. (1964). *Arch. Biochem. Biophys.* **106**, 49.
 Bonting, S. L., Simon, S. A. & Hawkins, N. M. (1961). *Arch. Biochem. Biophys.* **95**, 416.
 Burgen, A. S. B. & Emmelin, N. G. (1961). *Physiology of the Salivary Glands*, pp. 195–226. Baltimore: The Williams and Wilkins Co.
 Burton, K. (1956). *Biochem. J.* **62**, 315.
 Caiger, P., Morton, R. K., Filsell, O. H. & Jarrett, I. G. (1962). *Biochem. J.* **85**, 351.
 Charnock, J. S. & Post, R. L. (1963). *Aust. J. exp. Biol. med. Sci.* **41**, 547.
 Denton, D. A. (1957). *J. Physiol.* **140**, 129.
 Dickens, F. (1961). In *Haematin Enzymes*, p. 625. Ed. by Falk, J. E., Lemberg, R. & Morton, R. K. Oxford: Pergamon Press Ltd.
 Filsell, O. H., Jarrett, I. G., Atkinson, M. R., Caiger, P. & Morton, R. K. (1963). *Biochem. J.* **89**, 92.
 Hokin, L. E. & Hokin, M. R. (1963). *Annu. Rev. Biochem.* **32**, 553.
 Jarrett, I. G. & Filsell, O. H. (1964). *Biochem. J.* **91**, 217.
 Jarrett, I. G. & Potter, B. J. (1952). *Aust. J. exp. Biol. med. Sci.* **30**, 207.
 Judah, J. D. & Ahmed, K. (1964). *Biol. Rev.* **39**, 160.
 Kay, R. N. B. (1958). *J. Physiol.* **144**, 463.
 Kay, R. N. B. (1960). *J. Physiol.* **150**, 538.
 Lowenstein, J. M. (1961). *J. theoret. Biol.* **1**, 98.
 Reid, R. L. (1950). *Aust. J. agric. Res.* **1**, 338.
 Samson, F. E., Dick, H. C. & Balfour, W. M. (1964). *Life Sci.* **3**, 511.
 Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.
 Schwartz, A., Laseter, A. H. & Kraititz, L. (1963). *J. cell. comp. Physiol.* **62**, 193.
 Skou, J. C. (1957). *Biochim. biophys. Acta*, **23**, 394.
 Wilson, A. D. & Tribe, D. E. (1961). *Aust. J. agric. Res.* **12**, 1126.