Na Extrusion by the Sartorius of *Rana pipiens*

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ABSTRACT Sartorius muscles of *R. pipiens* may be enriched in sodium and depleted of potassium by prolonged soaking in the cold in K-free or low K Ringer's solution. Such K-depleted muscles take up potassium and extrude sodium when exposed to Ringer's solution containing 10 mM K at ordinary room temperature (*ca.* 20°C), with no dependence on external Na ion concentration in either phase within fairly wide limits.

Extrusion of excess fiber Na is demonstrated on single muscles using Na^{24}-loaded tissues. Rate of exchange of excess fiber Na does not differ significantly from the rate of exchange of Na normally present in muscle fibers.

Striated muscles of the common grass frog *R. pipiens* can, under appropriate conditions, extrude Na against an electrochemical gradient, meanwhile absorbing K in such a fashion that the sum of the two alkali ions remains approximately constant. This phenomenon of Na-K exchange has been studied extensively in isolated sartorius muscles, (*cf.* Steinbach, 1954, Stephenson, 1957, Frazier and Keynes, 1959, Conway, 1960).

In general, the results with amphibian striated muscle parallel those obtained with *in vitro* ion exchange studies on erythrocytes and other cellular systems. The amphibian sartorius, however, appears singularly unresponsive to metabolic inhibitors used singly (Frazier and Keynes, 1959).

Over a period of years evidence has been produced which may indicate specific differences, especially between *R. esculenta* and *R. pipiens*. A major question appears to be whether or not K-depleted, Na-enriched muscle can extrude excess Na when K is added to the bathing medium but with Na held constant (Carey *et al.*, 1959). It is claimed that with *R. esculenta* it is necessary to reduce Na coincidentally with the increase of K of the medium in order to obtain the Na extrusion. On the basis of this claim, "critical energy barriers" for sodium extrusion have been invoked (Conway, 1960).

This report will present data indicating the various conditions under which the sartorius muscles of *R. pipiens* can extrude Na after K depletion. Alteration of the sodium content of the environment is not essential for sodium extrusion by fibers of these muscles.

MATERIALS AND METHODS

Sartorius muscles were carefully dissected from grass frogs (*Rana pipiens*) obtained from a dealer in Wisconsin. Care was taken to choose animals appearing in good health but they were not fed nor otherwise given special attention. Experiments were carried out during the period November through May and hence all animals were probably collected in the fall. While all animals used in this series were *R. pipiens*, past work has shown that the findings apply to *R. palustris* as well.

As in earlier experiments, treatment of the muscles consisted of one or two phases. The first phase, the extraction phase, consisted of soaking the muscles in the cold (1–8°C) in about 200 times their volume of extracting medium for 20 to 24 hours without stirring. The second phase, the recovery period, consisted of placing the muscles in known volumes (usually about 200 times) of recovery fluid at 20–24°C (temperature ± 2°C for any given experiment). During recovery the vessels of recovery fluid were sometimes swirled gently by machine, other times not. No significant differences were noted in the results.

Muscle weights were determined at appropriate intervals after gentle blotting of the tissues on filter paper. Care was taken to handle the muscles by grasping tendinous portions with needle-tipped forceps.

Na and K analyses were performed with either the Beckman flame photometer or Coleman flame photometer on extracts prepared by heating the muscles to near boiling in 5 or 10 ml of H_2O to which 1 drop of glacial acetic acid had been added and then allowing the tubes to cool for at least 1 hour. Analysis of subsequently completely ashed residues indicated almost complete removal of Na and K by this method. The variable small remainder might be bound ion but is negligible in terms of the total ionic changes with which this report is concerned.

Cl was frequently determined electrometrically on the same extracts.

Rates of influx or efflux of ions were determined using Na^{24} and K^{42}, obtained as the chloride (in strong HCl) from the Oak Ridge National Laboratory. The isotopes were stated to be free from other radioactive contaminants and no further checks were made as routine. Care was taken to adjust the pH of all solutions to 7.4 ± 1 at the start of each experiment.

Relative K^{42} and Na^{24} contents of muscles were determined directly on the tissues by orienting the lightly blotted muscles on a specially designed wax surface planchette placed under a thin window counting tube. This method is quite successful for K^{42} since the ion is concentrated in the tissue but is much more variable for Na^{24} which is largely extracellular. In all cases, final determinations of radioactivity and total ion content of the muscle extract were made in order to express results, when desirable, in terms of specific activity or per cent exchange of the ion with the medium.

Before final weighing of the tissues for hot water extraction, the tendinous ends (*ca* 1 mm) were carefully clipped off. The response to the resultant mechanical stimulation was recorded but will not be reported in detail. In general, muscles extracted in the cold remained highly responsive. Muscles treated with K-enriched recovery medium (unless otherwise stated, containing 10 mM KCl) were, of course, unresponsive while muscles in K-free medium at room temperature for similar

periods of time retained their activity. The effects of K depletion on tension development will be reported later.

Both extracting and recovery fluids contained minimal concentrations of $CaCl_2$ (10^{-4} M), Na HCO_3 (10^{-4} M), and Na-phosphate buffer pH 7.4 (10^{-3} M). These minimal concentrations were chosen since it has been claimed that for *R. esculenta* it is necessary to have both fluids contain an ionic content similar to normal blood concentrations. No systematic study has been made to determine whether or not even the minimal amounts of ions other than Na, K, and Cl are necessary for the phenomena under investigation for *R. pipiens*. In the present series of observations neither glucose nor other metabolites was added.

pH values of common fluids were checked at the end of all experiments and were regarded as acceptable within the range 7.2–7.8.

RESULTS

A. *Inulin Space*

Tasker *et al.* (1959) have reported a comprehensive study of extracellular space in the sartorius muscle of the toad *Bufo marinus* and include a short review of the various values reported for frogs as well. Over a period of years values of inulin space in isolated sartorii of *R. pipiens* have been assembled in my laboratory. In general the results were consistent with the findings on toads. Values of inulin space range widely from about 15 to 40 per cent. As reported for toads however, there is a tendency for batches of frogs to show less variation in inulin spaces than does the total population taken over a period of years. Using trace amounts of C^{14}-labeled inulin, 8 muscles (4 pairs) showed an average space of 20 ± 1 per cent for the current batch of frogs. Older batches, with inulin space determined by the usual chemical method gave values as follows:—

(1) 31 ± 1 per cent (10 muscles)	(2) 25 ± 1.1 per cent (8 muscles)
28 ± 2 per cent (8 muscles)	20 ± 1 per cent (8 muscles)

For any precise measurement of extracellular ion levels it is certainly necessary, as pointed out by Tasker *et al.*, to measure inulin spaces on individual muscles. However, for approximate calculation, average results probably are not overly misleading. The value of 20 per cent extracellular space is used in any calculations made in this paper. This is lower than the values indicated by Johnson (1955) for sulfate space in fresh sartorii of *R. esculenta* but considerably higher than the frequently used 13 per cent value. Johnson (1956) working with *R. pipiens* found an average value of 18 per cent for inulin space of sartorii.

No consistent variations have been noted between muscles maintained in

high or low K Ringer's and the same inulin space is assumed for all conditions in which weight changes are small.

B. *The Influence of Potassium in the Medium on Extraction of Potassium from Muscle*

In order to demonstrate sodium extrusion by net changes in intracellular ion content it was found necessary to first enrich the muscle fibers with intracellular sodium. This Na enrichment has been shown to occur also during

TABLE I

Concentrations of Na and K in muscles soaked for 20 hours in K-free and low K (2 mM K) Ringer's and Ringer's containing 10 mM K. Temperature 1–5°C except for last 2 to 3 hours at room temperature (20°C). Solutions as indicated in text. Concentrations as millimols/kilogram final wet weight of tissue. Weight changes were negligible during soaking periods.

	Low K soaking	High K soaking	Difference
8 pairs, K-free and 10 mM K			
Na, tissue	84 ± 2.5	63 ± 1.5	21
			$p^*<0.0001$
K, tissue	32 ± 2.5	66 ± 1.8	34
			$p<0.0001$
5 pairs, 2 mol K and 10 mM K			
Na, tissue	58 ± 2.3	46 ± 3.5	12
			$p<0.005>0.025$
K, tissue	63 ± 3.1	78 ± 4.0	15
			$p<0.025>0.01$

*p values calculated by method of analysis of variance. I am indebted to Professor Thomas Park for assistance in this. Other indications of variations given as standard errors.

excitation and performance of work (*cf.* Fenn and Cobb, 1936). An easily controlled device for accomplishing what is apparently the same end result is to deplete the extracellular medium of K ion for longer periods of time, either by feeding K-free solutes to whole animal (Heppel, 1939) or by soaking isolated frog sartorii overnight in K-free Ringer's in the cold (Steinbach, 1940 a). Subsequent treatment with K-containing Ringer's at room temperatures results in Na extrusion and K uptake. Low temperature and a low concentration of external K in the soaking medium seem necessary and sufficient for the sodium enrichment process, increased K content of the medium and higher temperature for the recovery. The effects of K concentration and of temperature have been reported in some detail (Steinbach, 1954).

Isolated frog sartorii were soaked approximately 20 hours in the cold in

either K-free Ringer's or Ringer's with 10 mM K present. Table I shows marked excess of muscle Na in the muscles soaked in K-free Ringer's as compared with those in 10 mM K medium. Less loss of K is noted in 2 mM

TABLE II

EXTRUSION OF NA AND UPTAKE OF K BY K-DEPLETED MUSCLES

Extracted muscles, except where indicated, soaked 20 or more hours in K-free Ringer's in the cold and then were removed for analysis. Recovered muscles: other members of pairs given an additional 2 hours' treatment at *ca.* 20°C in Ringer's with Na and K concentrations in the media as indicated. Figures in parentheses indicate number of individual muscles, otherwise single determinations.

	Extracted	Recovered	Change on recovery
Extraction			
115 Na, 0 K			
Recovery 115, Na 10 K			
Na, tissue	70 ± 1.4 (12)	47 ± 2 (12)	−23($p<0.0001$)
K, tissue	49 ± 1.9 (12)	77 ± 2.4 (12)	+28($p<0.0001$)
Extraction			
125 Na, 0 K			
Recovery 125 Na, 10 K			
Na, tissue	75 ± 2.3 (12)	51 ± 4.3 (4)	−24($p<0.0001$)
K, tissue	51 ± 2.3 (12)	89 ± 1.5 (4)	+38($p<0.0001$)
Cl, tissue	37 ± 1.3 (12)	39 ± 1.2 (4)	−2($p>0.05$)
*Extraction			
110 Na, 0 K			
Recovery 130 Na, 10 K			
Na, tissue A	75	44	−31
B	81	49	−32
C	130	58	−72
K, tissue A	58	98	+40
B	60	87	+27
C	22	97	+75
Cl, tissue A	40	36	
B	38	43	
C	57	49	

* Extracted muscles also soaked 2 hours at room temperature in the 130 mM Na solution, K-free. The larger differences between extracted and recovered muscles probably reflect changes in extraction during this period.

K Ringer's soaked muscles than in K-free solutions although Na enrichment does take place in both.

It should be noted that the slightly higher value of Na + K for muscles from 10 mM K Ringer's as compared with those from K-free Ringer's can be almost entirely accounted for by the differing ionic strengths of the two solutions.

C. *Conditions for Demonstrating Na Extrusion and K Uptake by Isolated Sartorii of R. pipiens*

In the experiments first showing that isolated frog sartorii, enriched in Na by overnight extraction in K-free medium in the cold could extrude Na and pick up K, the extraction fluids contained 120 mM Na and 0 K, the recovery fluids 110 mM Na and 10 mM K, with ionic strength held constant (Steinbach, 1940 *a*). Subsequent work showed that the level of external Na in either extraction or recovery fluids was not critical (*cf.* Steinbach, 1954) provided

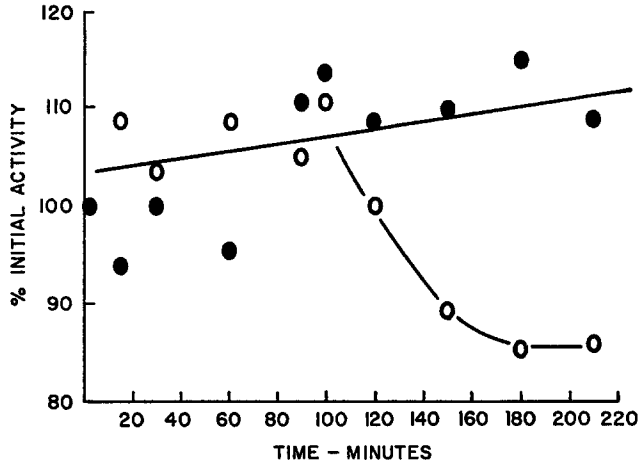

FIGURE 1. Muscles labeled with Na^{24} by extracting overnight in the cold in K-free Ringer's, Na^{24} added. Muscles then removed to room temperature in the same solutions and radioactivity followed as described in text. With one set (circles) KCl to final concentration of 10 mM was added with minimal dilution (1 ml 1 M KCl/100 ml solution), 1 ml H_2O per 100 ml being added to other set (solid circles). Sodium extrusion from fibers is illustrated by drop in Na^{24} activity of muscles, tissue spaces remaining constant.

that there was sufficient Na in the medium during the extraction period. Further data on this point are presented in Table II.

Isolated sartorius muscles previously depleted of K and enriched with Na can extrude Na and take up K from recovery medium of varying Na concentration providing (1) the temperature is high enough and (2) K is present in sufficient concentration in the recovery medium. It is not necessary to shock the tissue by a high Na–low Na sequence (compare Conway, 1960).

Na extrusion may be illustrated dramatically by adding K to Na^{24}-labeled muscles in the presence of the tracer Na (Fig. 1). Muscles were soaked overnight in K-free Ringer's containing Na^{24}. These muscles, with soaking solution, were then brought to room temperature and muscle radioactivity measured by placing them on a waxed glass plate directly under a thin window counter.

This method, due to unavoidable variations in the amount of extraneous soaking fluid carried with the muscle is subject to large errors, but in general, muscle radioactivity remained reasonably constant for the 80 minute preliminary period. KCl was then added (0.1 ml 1 M KCl/100 ml of K-free Na^{24} Ringer's) and the counting continued. Following a short interval of about the time noted for washout of extracellular space (Levi and Ussing, 1948) the radioactivity of the recovering muscle drops rapidly and smoothly, the control muscle remaining at full radioactive level. The kinetics of the extrusion process is similar to that previously reported (*cf.* Steinbach, 1954, Frazier and Keynes, 1959). Pertinent analytical data for the experiment are given in Table III.

TABLE III

DATA ON MUSCLES FROM EXPERIMENT ILLUSTRATED IN FIG. 1

Concentrations in millimols/kilogram wet weight tissue. Specific activities as per cent specific activities of medium.

	Extracted	Recovered	Change
Na tissue	70	44	−26
K tissue	54	83	+29
Specific activity, *per cent*	97	92	

D. *Exchangeability of Tissue Na*

Muscles depleted of K and loaded with Na by prolonged soaking in K-free Ringer's have a large component of intracellular Na. Assuming 20 per cent extracellular space, the values in Table I indicate approximately 60 mM Na intracellular and 60 mM K in K-depleted muscles as opposed to roughly 30 mM Na and 90 mM K in recovered muscles. Thus intracellular Na is at least doubled during K depletion while intracellular K is reduced by one-third. Normal muscle Na is much more rapidly exchangeable than K and there is some evidence that excess fiber Na is more slowly exchangeable than that normally present (*cf.* Harris (1960) for discussion).

Four pairs of sartorii were soaked 23 hours in the cold, one member of each pair in K-free Ringer's, the other in 10 mM K Ringer's. All muscles were then transferred to K-free Ringer's at room temperature, allowed to equilibrate for 60 minutes, and then treated with tracer amounts of Na^{24}. The time course of entry of Na^{24} into the separate muscles was then followed as described earlier. At the end of 3 hours the muscles were weighed, extracted, and radioactivity and Na and K content determined on the extracts. Calculating from the per cent of exchange determined on the extracts, the time courses of exchange are plotted in Fig. 2. Table IV summarizes the essential data. There is indicated a slightly lower rate of exchange of Na^{24} in the high Na

muscles but the differences between rates in low and high Na muscles are not great. Since, under the same conditions (low external K) K exchanges very slowly, it is clear that the excess Na is not subject to the same restrictions as that of the K displaced during enrichment with Na.

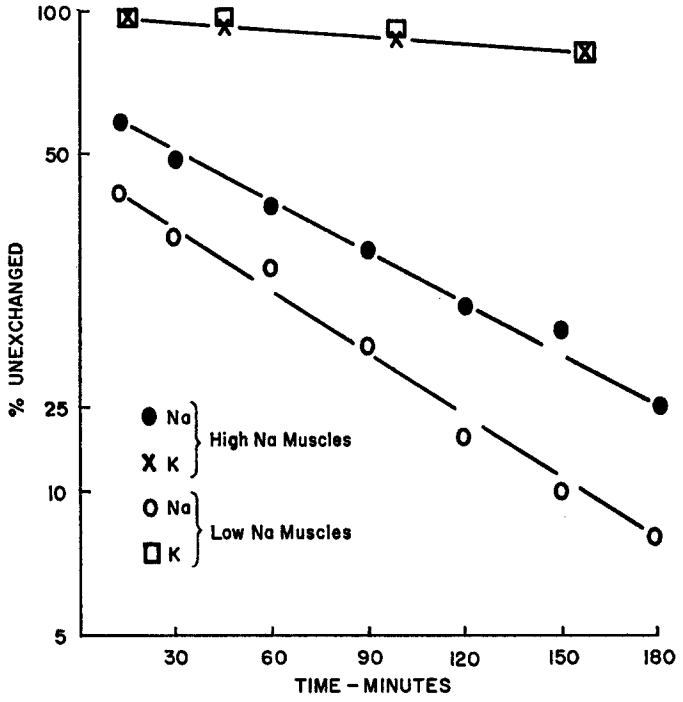

FIGURE 2. Influx of Na^{24} and K^{42} in muscles either enriched in Na by soaking in K-free Ringer's or with normal Na by comparable soaking in Ringer's containing 10 mM K (see text).

TABLE IV

Frog sartorii immersed 23 hours in the cold in K-free Ringer's or 10 mM K-Ringer's. All muscles then transferred to K-free Ringer's at 24°C for 1 hour, then to Na^{24}-K-free Ringer's for 3 hours. Concentrations in millimols/kilogram wet weight of tissue. Average weight at end of K-free immersion, 97 per cent of weight at end of cold extraction. Per cent exchange refers to specific activity muscle/specific activity medium × 100. Figures are averages of two determinations. Figures in parentheses give exchange of K^{42} under comparable experimental conditions but with 2 mM K in medium at room temperature.

	High Na muscles	Low Na muscles
K concentration	30	70
Na concentration	86	61
Na per cent exchange	85	92
(K^{42} per cent exchange) (5 hrs.)	(28)	(25)

Comparable experiments (Table IV, Fig. 2) indicate that the K of low K muscles exchanges with K^{42} of the environment at the same slow rate as previously noted for normal muscles (*cf.* Harris, 1960) and as compared with muscles held overnight in 10 mM K Ringer's followed by equilibration in low K Ringer's.

DISCUSSION

In striated muscle fibers, relatively stable non-growing systems, Na and K ions exchange on a reciprocal basis within fairly wide limits, behaving as though the internal anionic component capable of associating with mobile cations remains fixed at a level balancing the ionic strength of the normal external medium. This state of affairs is not always noted in other systems less specialized in functions such as liver cells, ascites tumor cells, etc. In the case of amphibian striated muscle the Na-K exchange appears to approximate a one to one basis for the relatively large net changes discussed in this paper. Whether or not this indicates an intimate coupling of K and Na handling mechanisms remains a question, however, since the fixed anion quality of the interior of the fiber backed up by any selective Na extrusion mechanism would give essentially the same result.

With respect to the outward extrusion of Na from Na-enriched sartorii (Steinbach, 1940 *a*), the general phenomenon has been amply confirmed. The basic requirements for demonstrating net outward transport of Na from muscles of *R. pipiens* are very simple. Na-enriched muscles may be prepared by prolonged soaking in the cold in K-free (or low K) Ringer's. The detailed requirements for this extraction process have not been studied. Such Na-enriched muscles will extrude excess Na, and take up K in exchange provided (1) the temperature is raised and (2) adequate K ion is in the recovery medium. A dependence on other constituents in the environment has not been demonstrated, Na extrusion taking place when muscles are bathed in salt solution buffered with phosphate or bicarbonate or both, and with or without specific concentrations of Ca or Mg ions present. With muscles as used in these studies there is also a relative independence of the Na extrusion of the external Na concentration during recovery. As first demonstrated, Na extrusion was induced by substituting K in the recovery fluid for some of the Na of the salt solution used for preliminary extraction (Steinbach, 1940 *a*, Stephenson, 1957). That is, extraction was in a fluid Na = 120, K = 0. Recovery fluids contained Na = 110, K = 10. Later work showed that essentially the same results were obtained with Na concentrations held the same in both soaking and recovery fluids, or with Na concentration drastically reduced by substitution of choline chloride for NaCl (Steinbach, 1952 and references cited). The present experiments confirm these findings for *R. pipiens*. It has

been claimed that with muscles from some species or varieties of frogs it is *necessary* to reduce Na in the recovery fluid (Conway, 1960), following the pattern approximately of the earlier experiments (Steinbach, 1940 *a*). This may involve a species difference but such an explanation requires independent confirmation. Conway (1960) has recently ascribed the failure noted by Frazier and Keynes (1959) to confirm the claimed CN sensitivity of Na extrusion to "different metabolic states." Conway (1960) did not specify the species of frogs used.

Shaw and Simon (1955) and van der Kloot (1956) have noted that Na extrusion against a gradient can be demonstrated in toad and frog sartorii when the salt content of the external medium is halved by dilution with isotonic sucrose. This process is reported as not sensitive to cyanide.

The general picture for the Na extrusion process from Na-enriched sartorii of *R. pipiens* thus is that of an internally controlled mechanism except for the necessity of K ions in the external medium. The rate of extrusion is related to the internal concentration of Na and is highly dependent on temperature (Steinbach, 1954). In fresh muscles Keynes and Swan (1959) report a marked effect of external Na concentration on Na efflux, this effect disappearing on *in vitro* storage.

There is no direct evidence to show whether or not excess Na in the muscle fiber occupies the same sites as the K that is lost in the exchange process. The fact that excess Na exchanges with environmental Na at a much faster rate than does K would argue for some difference in the intracellular condition if not location of the two species of ions. It has been demonstrated that the ionic changes found in muscles immersed in hypotonic and hypertonic solutions are consistent with the idea that an appreciable portion of the muscle fiber is freely permeable to all univalent ions (Steinbach, 1947). Recently the endoplasmic reticulum system has been suggested as the freely diffusible chamber (Simon, 1959). One difficulty is, of course, the necessity for invoking large scale changes in "compartment" size, if distributions of Na and K are assumed to be limited by morphologically distant intracellular areas.

From a comparative point of view, it is a remarkable fact that the potassium concentration, calculated on a fiber water basis, is nearly the same for almost all muscles that have been tested, whether they come from animals with body fluids of as low ionic strength as that of the frog or fresh water crayfish or from an environment of as high ionic strength as sea water (Table V, Steinbach, 1947 and Robertson, 1957). Thus any mechanisms proposed for ionic regulation in muscle tissues must account for the maintainence of a constant level of K under normal conditions regardless of the ionic strength of the normal environment. Ratios of intracellular/extracellular concentrations of Na, K, and Cl are hgihly variable between the different groups of animals,

and only the K concentration is relatively constant. It should be noted that muscles of fresh water clams constitute exceptions, some forms maintaining an internal K concentration only about one tenth that of the average value shown in Table I. This is about the level noted in fresh water protozoa and may indicate a basic level of active potassium uptake, unassisted by mechanisms for extruding other ions in the face of high external concentrations.

TABLE V

CALCULATED VALUES FOR NA AND K IN MUSCLE FIBER WATER BAND ON DATA FROM VARIOUS SOURCES

	Fiber H_2O		$\frac{Na_i}{Na_o}$	$\frac{K_i}{K_o}$
	Na	K		
Rat thigh*	8.4	185	0.056	41
Rabbit uterus*	101	101	0.69	24
Cat uterus*	143	120	0.90	32
Chick‡	8.5	147	0.05	20
Frog‡	11.4	125	0.11	50
Romalea§	3.3	150	0.05	8
Periplaneta	15.0	120	0.14	7
Mytilus‡	121	137	0.24	7
Eriocheir‖	33	136	0.07	18
Carcinus‖	54	112	0.11	9
Nephrops‖	27	177	0.05	21
Phascolosoma	87	174	0.21	11
Astacus¶	21	145	0.10	32

* Daniel and Daniel (1957).
‡ Barlow and Mavery (1954).
§ Tobias (1948).
‖ Robertson (1957).
¶ Bryan (1960).

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