Congr. Biochem., 7th, Tokyo 5, 923 (1967).

7. Yeh, S. D. J. and Shils, M. E., Proc. Soc. Exptl. Biol. Med. 121, 729 (1966).

8. Strauss, E. W., Wilson, T. H., and Hotchkiss, A., Am. J. Physiol. 198, 103 (1960).

9. Yeh, S. D. J. and Shils, M. E., Federation Proc. 27, 359 (1968).

10. Siegel, M. R. and Sisler, H. D., Biochim. Biophys. Acta 87, 70 (1964).

11. Gräsbeck, R., Simons, K., and Sinkkonen, I., Biochim. Biophys. Acta 127, 47 (1966).

12. Frentz, G. D., Miller, O. N., and Hansen, H. J., Federation Proc. 27, 359 (1968).

Received July 30, 1968. P.S.E.B.M., 1969, Vol. 130.

Effect of External Potassium and Ouabain on Sodium Efflux from Frog Sartorius Muscle* (33769)

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It is well known that the efflux of Na ions from frog skeletal muscle depends upon the external K concentration (1). It is also well established that this K stimulated efflux of Na, as well as the net exchange of Na and K ions in this tissue, is inhibited by the cardiac glycoside ouabain (G-strophanthin) and its aglycone strophanthidin (2–7). The inhibitory effects of cardiac glycosides and aglycones on Na and K transport appear to be quite general and have been demonstrated in a large variety of cells and tissues (8).

In several instances, it was found that the inhibitory effects of cardiac glycosides and related substances can be reversed by increasing the external K concentration (9–12). It has been suggested that, in some cells at least, cardiac glycosides in low concentrations compete with K for a site in the cell membrane which is implicated in the active transport of Na, though quantitative demonstration of a competitive relationship has proved difficult (9–11).

Despite the widespread use of cardiac glycosides in studies of Na and K transport in frog skeletal muscle, comparatively few studies were made of the interrelationship between the K. concentration of the external medium and the inhibitory effect of these substances. The present paper is concerned with some aspects of this interrelationship.

Materials and Methods. Pairs of freshly dissected sartorius muscles weighing about 70–80 mg from a single frog (*Rana pipiens*) were immersed overnight at 5° in K-free Ringer's containing 120 meq of Na/liter. The Na was labeled with a tracer amount of ^{24}Na (obtained as $^{24}NaCl$ in HCl solution from the Oak Ridge National Laboratory). Following this immersion, the muscles were carefully blotted, weighed, and washed out for 3 hr at room temperature (K25°) in 5-ml aliquots of nonradioactive Ringer's solutions containing 104 meq of Na/liter. These aliquots were subsequently assayed for ^{24}Na using a well scintillation detector.

The washout procedure for each pair of companion muscles was as follows. One member of the pair was kept in K-free Ringer's throughout. Its companion was first washed out in an identical K-free solution for 1 hr. During the second hour it was transferred to successive aliquots of a solution containing 104 meq of Na/liter together with a constant amount of KCl. Finally it was washed out for a third hour in a solution containing 1×10^{-6} *M* ouabain[1] but identical in other re-

* Supported by U. S. Public Health Service Grant GM-10971 and by facilities provided by Indiana University Heart Research Center Grant H-6308 from the National Heart Institute, U. S. Public Health Service. Statistical computations were performed at the Research Computation Center, Indiana University Medical Center, and supported by U. S. Public Health Service Grant FR 00162.

[1] This concentration of ouabain was chosen because, although it is high enough to cause virtually

complete inhibition of active Na transport in frog sartorius muscle (2), it lies within the range of concentrations at which reversal of inhibition by external K has been observed with other tissues (9, 11, 12).

spects with the washout solution used during the second hour. The KCl concentrations used in these experiments ranged from 2.5 to 15.0 m*M*. Three experiments were carried out with each external KCl concentration employed. To maintain constant osmolarity in the soaking solutions appropriate amounts of glucose were added.

At the end of the washout period the muscles were blotted free of adhering solution, weighed again, and dissolved by gentle warming in 1 ml of concentrated HNO_3. Aliquots of the resulting solutions (diluted to 5 ml with water) were counted to determine the residual radioactivity in the muscles. By successively summing the counts back to the beginning of the washout, the relative amounts of radioactivity remaining in the muscles at any given time during washout could be determined.

All the soaking solutions used were of the bicarbonate Ringer's type originally devised by Boyle and Conway (13) for frog muscle. Before use the solutions were brought to pH 7.4 by gassing them with a mixture of 95% oxygen and 5% carbon dioxide. "Analytical Reagent" grade materials were used throughout in making up these solutions.

The ouabain used was a highly purified sample and was the gift of Eli Lilly and Company, Indianapolis.

Results and Discussion. The data obtained for the washout of ^{24}Na at two different external K concentrations (2.5 and 8 m*M*) are shown in Figs. 1 and 2. In both figures the logarithm of the average ^{24}Na content of three pairs of companion muscles (expressed as a percentage of the initial ^{24}Na content) is plotted as a function of time. In each case one member of each pair was washed out in K-free Ringer's throughout the experiment, its companion being transferred from K-free Ringer's to a second Ringer's solution containing K, and subsequently to a third solution containing K plus ouabain as described

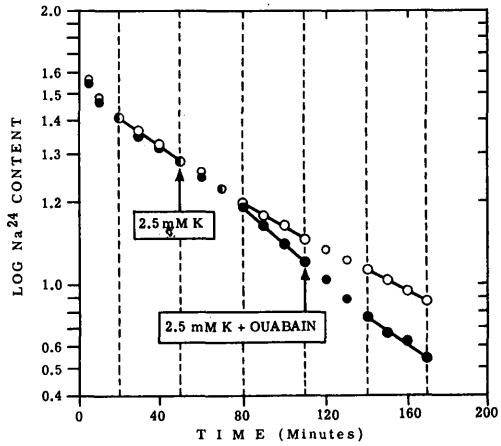

FIG. 1. Loss of ^{24}Na from companion muscles loaded overnight at 5° in K-free Ringer's containing 120 m*M* Na labeled with ^{24}Na and subsequently washed out in inactive Ringer's containing 104 m*M* Na. One muscle (○) washed out in K-free Ringer's throughout the experiment. Its companion (●) was first washed out in K-free Ringer's then in Ringer's containing 2.5 m*M* K and finally in Ringer's containing 2.5 m*M* K plus 1×10^{-6} *M* ouabain as indicated. Results shown are average values for 3 experiments. Logarithm of ^{24}Na content of muscles (expressed as a percentage of their initial ^{24}Na content) plotted as a function of time.

above and as indicatel in Figs. 1 and 2. In both figures the increase in the rate of loss of ^{24}Na following transfer to a solution containing K can clearly be seen, the relative increase observed being much greater in the presence of 8 m*M* K than in the presence of 2.5 m*M* K. It is also apparent from Fig. 1 that there is a substantial reduction in the rate of loss of ^{24}Na following transfer of the muscles from a solution containing 2.5 m*M* K to one containing 2.5 m*M* K plus ouabain. A similar but smaller reduction in the rate of loss of ^{24}Na is suggested by the data illustrated in Fig. 2 but, as shown below, this apparent reduction is not significant at the 0.01 confidence level.

Essentially similar results to those shown in Figs. 1 and 2 were obtained with 5, 10, and 15 m*M* *K*. Figures 1 and 2 also serve to illustrate the methods employed for the quantitative evaluation of the stimulation by external K of ^{24}Na efflux and for the effect of ouabain on this K stimulated efflux. Consid-

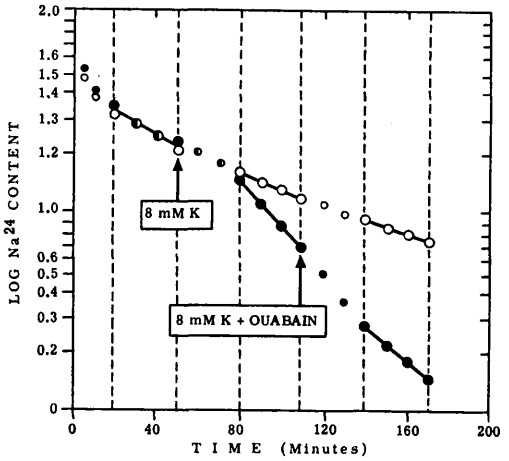

FIG. 2. Washout of ^{24}Na from companion muscles labeled as in Fig. 1. One muscle (O) in K-free Ringer's throughout; its companion transferred as indicated from K-free Ringer's to Ringer's containing 8 m*M* K and finally to Ringer's containing 8 m*M* K plus 1×10^{-6} *M* ouabain; average of three experiments; results plotted as in Fig. 1.

ering first the washout curves obtained in K-free media, it is seen that, under these conditions, the efflux of ^{24}Na shows a fast phase during the first 15–20 min of washout. This fast phase probably represents the washout of initial radioactivity from the extracellular space. From the Na efflux data reported in the literature (1) for muscles of the size used in these experiments, it can readily be shown that the rate constant for Na exchange between the extracellular space and the bathing medium is some 40–50 times greater than the rate constants reported in the present paper for the washout period between 20 and 50 min (Table I). Therefore, the contribution of the initial fast component of washout to rate constants determined after the first 20 min of washout can be expected to be negligible. The use of an equation of the form of Eq. (1) to determine such rate constants, appears therefore to be justified. The fast component of washout is followed by a slower phase of ^{24}Na loss. Inspection of the washout curves for the control muscles shown in Figs. 1 and 2 indicates that this second phase cannot be characterized by a

TABLE I. Effect of External K and of Ouabain on the Rate of Loss of ^{24}Na from Frog Sartorius Muscles.[a]

Group	External K (m*M*)	Rate constant for ^{24}Na loss (min^{-1}) I 20–50 min	*p*	II 80–110 min	*p*	III 140–180 min	*p*
1 A	2.5	0.0124 ± 0.0010		0.0105 ± 0.0010		0.0090 ± 0.0009	
B		0.0121 ± 0.0010	>0.05	0.0157 ± 0.0007	<0.01	0.0122 ± 0.0005	>0.05
2 A	5.0	0.0151 ± 0.0006		0.0125 ± 0.0003		0.0107 ± 0.0006	
B		0.0149 ± 0.0003	>0.05	0.0231 ± 0.0005	<0.01	0.0171 ± 0.0004	<0.01
3 A	8.0	0.0117 ± 0.0002		0.0095 ± 0.0002		0.0079 ± 0.0001	
B		0.0125 ± 0.0003	0.02–0.05	0.0272 ± 0.0014	<0.01	0.0198 ± 0.0006	<0.01
4 A	10.0	0.0119 ± 0.0005		0.0101 ± 0.0003		0.0098 ± 0.0005	
B		0.0116 ± 0.0004	>0.05	0.0313 ± 0.0008	<0.01	0.0262 ± 0.0031	<0.01
5 A	15.0	0.0109 ± 0.0003		0.0079 ± 0.0003		0.0068 ± 0.0002	
B		0.0116 ± 0.0007	>0.05	0.0425 ± 0.0020	<0.01	0.0294 ± 0.0014	<0.01

[a] Average rate constants (± SE) are given for each group of muscles for the indicated time periods (measured from the start of the washout procedure). Each group includes 3 pairs of companion muscles. One member of each pair (A) was kept in K-free Ringer's throughout. Its companion (B) was exposed to K-free Ringer's during the first time interval (20–50 min). During the second time interval (80–110 min) it was immersed in Ringer's containing K in the amount indicated and, during the final interval (140–180 min) in Ringer's containing the same amount of K plus 1×10^{-6} *M* ouabain. The *p* values for the comparison of A and B muscles in each group are based on Satterthwaite's adjusted *t* test for inhomogeneous variances.

single first-order rate constant during the entire period of washout. The presence of higher order kinetic components in the total washout curve was confirmed by statistical analysis. However, it was found that for relatively short segments of the washout curve (30–40 min) the deviations from linearity were not significant.[2] Similarly, in the washout data for muscles which were transferred from K-free solutions to solutions containing K and, subsequently, to solutions containing K plus ouabain, it was found that, if allowance is made for the establishment of steady state conditions following each transfer,[3] the efflux of ^{24}Na under these conditions obeys first-order kinetics at the 0.05 confidence level.

Evaluation of the washout data in terms of first-order rate constants was therefore carried out as follows: Linear regression equations were computed for each group of 3 muscles exposed to K and to K plus ouabain during the time intervals 20–50, 80–140, and 140–180 min (both limiting times being included in each case) and for the corresponding groups of control muscles during the same time intervals. From these equations the corresponding first-order rate constants were computed from the relationship

$$k_t = -d \ln [^{24}Na]/dt = -2.303\ b, \quad (1)$$

where k_t is the rate constant, $[^{24}Na]$ is the percentage of ^{24}Na remaining in the muscle at time t and b is the regression coefficient of $\log_{10} [^{24}Na]$ on t.

It is evident from Figs. 1 and 2 that the time intervals chosen correspond to steady state efflux conditions during (i) simultaneous exposure of both groups of muscles to K-free Ringer's, (ii) exposure of one group to K and the other to K-free Ringer's during the same time, and (iii) exposure of one group to K plus ouabain and simultaneous exposure of the other to K-free Ringer's. Comparisons between the rate constants obtained under these conditions were made using Satterthwaite's adjusted t test for the comparison of two means when their variances are not homogenous (14).

The results obtained are summarized in Table I. Several points of interest emerge from a consideration of these results. Firstly, as shown, the rate of ^{24}Na efflux into K-free Ringer's did not remain constant throughout the washout period but declined progressively with time. This is apparent from a comparison of the average rate constants obtained for the muscles of subgroup A in each group of muscles during the time intervals chosen (Table I). Secondly, the rate constants for the muscles in subgroups A and B of each group, with one exception, did not differ significantly at the 0.05 confidence level during efflux into K-free Ringer's. In one case (external K = 8 mM) the rate constants did differ significantly at the 0.05 level but were not significantly different at the 0.01 confidence level. Thus it would appear that, even with the relatively small number of muscles used in these experiments, the average rate constant obtained by grouping together the results obtained with one member of a pair of companion muscles may justifiably be used as a control value in assessing the effect of various experimental conditions on the efflux of ^{24}Na from the contralateral muscles from the same animals.

Table I further shows that, in all cases, external K caused a significant increase ($p < 0.01$) in the rate of loss of ^{24}Na (column II of Table I). Also, when the external K concentration was relatively low (2.5 mM), 1×10^{-6} M ouabain completely inhibited the increase efflux of ^{24}Na due to K ($p > 0.05$ for the rate constants for subgroups A and B of group 1 during the time interval 140–180 min). With higher K concentrations complete inhibition was not observed (column III of Table I).

The stimulating effect of external K on

[2] Writing y for the logarithm of the relative amount of ^{24}Na remaining at time x from the beginning of the washout procedure it was found that, for 40-min segments of the slow phase of washout, $p < 0.05$ for second and higher order terms in the regression equation: $y = a + bx + cx^2 + \ldots$.

[3] This was done by omitting from the computations the values between 0 and 20 min, 50 and 80 min, and 110 and 140 min as indicated by the broken lines in Figs. 1 and 2.

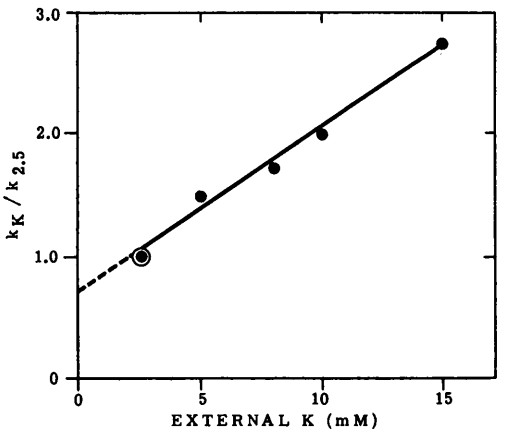

FIG. 3. The effect of external K concentration on the efflux of ^{24}Na from frog sartorius muscle. The ratio of the rate constant (k) at the external K concentrations indicated to the rate constant in a medium containing 2.5 mM K is plotted as a function of external K concentration in mM. Apart from the point corresponding to 2.5 mM external K (⊙), each point represents the average of three independent estimates of the ratio $k_K/k_{2.5}$. The line of regression shown was computed for the points corresponding to external K concentrations of 5–15 mM.

^{24}Na efflux is further examined in Fig. 3. In this figure the ratio of the rate constant k for ^{24}Na loss in high K media to that observed in media containing the normal plasma K concentration of 2.5 mM ($k_{2.5}$) is plotted as a function of external K concentration. Figure 3 shows that this ratio increased linearly with external K concentration. The sample regression equation for the data shown in Fig. 3 is

$$k/k_{2.5} = 0.713 + 0.126\,[K_e], \qquad (2)$$

where $[K_e]$ is the external K concentration in mM ($S_b = \pm 0.011 : r = 0.993$). The ratio obtained from this equation for $[K_e] = 0$ (0.713), is in good agreement with the average value for the same ratio (0.67) calculated from the data of Table I, suggesting that the linear relationship illustrated in Fig. 3 also extends to external K concentrations below 2.5 mM. These results, obtained following a loading procedure in which the average Na content of the muscles is increased from the normal value of 23.9 mM to about 56 mM (15, 16) are in sharp contrast to the results reported by Horowicz and Gerber (7). These authors examined the effect of external K concentration on the efflux of Na from small bundles of fibers from frog semitendinosus muscle labeled with ^{24}Na under conditions which did not results in significant increases in total muscle Na. Under these conditions a plot of the results obtained, similar to that shown in Fig. 3, gave an S-shaped curve. With external K concentrations from 2.5 to 7.5 mM little change was observed in the rate of ^{24}Na efflux. Between 7.5 and 10 mM K, ^{24}Na efflux increased sharply with increasing external K concentration. Above 10 mM K the rate of increase in ^{24}Na efflux with increasing external K concentration declined markedly.

These results have been interpreted in terms of a mechanism in which the activity of the Na pump in the muscle fiber is controlled by the transmembrane potential. Specifically, it has been suggested (7) that marked stimulation of Na pump activity only occurs at or above a certain threshold level of depolarization (about 22 mV). The results shown in Fig. 3, and in particular the absence of a detectable threshold for the effect of external K on ^{24}Na efflux in the present experiments, strongly suggest that the mechanisms controlling ^{24}Na efflux in Na loaded fibers are different, quantitatively at least, from those which are operative in freshly dissected muscles.

Inspection of the results given in column III of Table I shows that, while 1×10^{-6} M ouabain completely inhibited the increased ^{24}Na efflux found with an external K concentration of 2.5 mM ($p > 0.05$ for the rate coefficients found for subgroups A and B of group 1 during the washout interval 140–180 min), the inhibitory effect of the glycoside is incomplete at higher external K concentrations ($p < 0.01$ for the rate coefficients in subgroups A and B in all cases) and appears to decrease with increasing external K concentration.

Because of the scatter in the results, it is not apparent from Table I whether the inhibitory effect of ouabain is completely re-

TABLE II. Effect of External K Concentration on the Inhibition of ^{24}Na Efflux by Ouabain.[a]

External K (mM)	k_B/k_A 80–110 min (without ouabain)	k_B/k_A 140–180 min (1×10^{-6} M ouabain)	t	p
5.0	1.85 ± 0.10	1.60 ± 0.09	1.84	<0.05
8.0	2.86 ± 0.31	2.50 ± 0.16	1.04	>0.05
10.0	3.10 ± 0.21	2.68 ± 0.64	0.62	>0.05
15.0	5.38 ± 0.97	4.36 ± 0.93	0.76	>0.05

[a] Ratio of rate coefficients (k_B/k_A) for muscles included in subgroups B and A of Table I (± SE) are given for the washout intervals 80–110 and 140–180 min. Differences between corresponding ratios tested for significance at the 0.05 confidence level using Satterthwaite's t test.

versed by K ions in sufficiently high concentration or whether a partial reversal only is obtained. This point is explored further in Table II. This table shows a statistical comparison (based in Satterthwaite's modification of Student's t test) between the ratios of the rate coefficients for subgroups A and B during the time interval 80–110 min and the corresponding ratio during the time interval 140–180 min, for external K concentrations ranging from 5 through 15 m. Because of the relatively small number of observations involved, this comparison is of necessity somewhat approximate. However, the data of Table II show rather clearly that, while there is still some inhibition (at the 0.05 confidence level) of Na efflux by ouabain in the presence of 5 mM K, the inhibitory effect of this compound is completely abolished in the presence of 8 mM K or more ($p > 0.05$). These results are compatible with a competitive relationship between ouabain, in the concentration used in these experiments, and external K ions.

Summary. Using paired muscles from the same animal, the effect of external K, in concentrations ranging from 2.5 to 15 mM, on the efflux of ^{24}Na from frog sartorius muscles (previously enriched in Na by overnight soaking at 5° in a K-free medium) was investigated. In agreement with the results reported by others, it was found that external K ions, over the concentration range studied, increased the rate of loss of ^{24}Na from the muscle fibers. The ratio of the rate coefficient for ^{24}Na efflux in high K media to that observed in a normal K medium (2.5 mM) was a linear function of external K concentration throughout the concentration range investigated. In media containing 2.5 mM K, ouabain (1×10^{-6} M) completely inhibited the stimulatory effect of K on Na efflux. With 5 mM K in the medium, inhibition was incomplete and, in media containing 8 mM K or more, no significant inhibition by ouabain of the efflux of ^{24}Na was noted. These results are compatible with a competitive inhibition by ouabain, at the concentration used, of the stimulating effect of K on Na efflux in muscle.

The technical assistance of Rita Virmani is gratefully acknowledged. The author's thanks are due to Dr. P. L. Yu of the Institute of Psychiatric Research, Indiana University Medical Center, for much helpful advice and assistance in the statistical computations.

1. Harris, E. J., "Transport and Accumulation in Biological Systems," 2nd ed., Chapt. 7. Macmillan (Pergamon), New York (1960).

2. Johnson, J. A., Am. J. Physiol. **187**, 328 (1956).

3. Edwards, E. and Harris, E. J., J. Physiol. **135**, 567 (1957).

4. Carey, M. J., Conway, E. J., and Kernan, R. P., J. Physiol. **148**, 51 (1959).

5. Conway, E. J., Kernan, R. P., and Zadunaisky, J., Am. J. Physiol. **155**, 263 (1961).

6. Keynes, R. D., J. Physiol. **178**, 305 (1965).

7. Horowicz, P. and Gerber, C. J., J. Gen. Physiol. **48**, 489 (1965).

8. Ussing, H. H., *in* "Handbuch der Experimentellen Pharmacologie," Vol. 13, p. 1. Springer, Berlin (1960).

9. Glynn, I. M., J. Physiol. **136**, 148 (1957).

10. Glynn, I. M., and Dunham, E. T., J. Physiol. **156**, 274 (1961).

11. Page, E., Goerke, R. J., and Storm, S. R., J.

Gen. Physiol. 47, 531 (1964).
12. Armstrong, W. McD., Am. J. Physiol. 206, 469 (1964).
13. Boyle, P. J., and Conway, E. J., J. Physiol. 100, 1 (1941).
14. Li, J. R. C., "Statistical Inference," Vol. 1, p. 143. Edwards, Ann Arbor, Michigan (1964).
15. Armstrong, W. McD., Am. J. Physiol. 208, 61 (1965).
16. Conway, E. J., Physiol. Rev. 37, 84 (1957).

Received Sept. 4, 1968. P.S.E.B.M., 1969, Vol. 130.

Dietary Effects on the Need for Glycine by the Chick (33770)

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While the need for glycine by the growing chick was shown by Almquist *et al.* (1), Akrabawi and Kratzer (2) showed that the requirement depends on the level of arginine in 24% casein diets and the presence of L-serine in amino acid mixture diets. Since serine hydroxymethyltransferase (E.C. 2.1. 2.1, L-serine tetrahydrofolate 5,-10-hydroxymethyltransferase) catalyzes the interconversion of glycine–serine in the liver (3–5), we wished to determine whether dietary arginine changes the glycine requirement by influencing the activity of this enzyme. This reaction was also studied by observing the effect of 2-levels of L-serine in the amino acid mixture diet on the plasma level of glycine and other amino acids of 2-week-old chicks.

Because L-serine was shown (2) to support growth of chicks equal to that of glycine when an amino acid mixture diet was fed, but not with a casein diet, there is a question of whether adequate L-serine was used. This was studied by comparing the response of chicks fed added glycine to those fed a higher level of L-serine in a 24% casein diet.

Materials and Methods. Day-old Arbor Acre chicks were weighed individually and put into groups of comparable weight and weight distribution. They were housed in electrically heated batteries with raised wire floors and were supplied feed and water *ad libitum*. The experimental diets were based on corn starch and 24% casein or 26.2% amino acid mixture (2). Both diets contained soybean oil, glycerol, cellulose, vitamin mixture, and chromic bread at levels of 5, 5, 3, 1.1, and 1% respectively. They also contained enought calcium, phosphorous, and other minerals to satisfy the dietary requirements. In Expts. 1 and 2, two groups of chicks were raised on a 24% casein diet with 0.4 or 1.2% L-arginine·HCl. After 2 weeks, 3 chicks from each group were killed by neck dislocation. The liver from each chick was quickly removed, weighed, and frozen on dry ice. The livers, each wrapped individually were stored at —10° for not longer than 1 week before assay. The activity of serine hydroxymethyltransferase was determined on a crude homogenate according to the method of Scrimgeour and Huennekens (6). A 1-g liver sample was homogenized in 3 ml of 0.1 *M* phosphate buffer (pH 7.5). Two-tenths ml of the crude homogenate was added to the reaction mixture. A 0.3-ml aliquot of the supernatant was used in formaldehyde assay according to the method of Nash (7).

In Expts. 3 and 5, different levels of glycine and L-serine were used. Nigrogen retention was determined according to the methods of Hill *et al.* (8) and Hill and Anderson (9) on excreta collected during days 11 through 13 from the onset of feeding. Body weight gains and feed efficiency were analyzed statistically according to Snedecor (10). In Expt. 4, an amino acid mixture diet containing 2.2% L-arginine·HCl was fed with different levels of glycine or L-serine. A sample of 0.5 ml of blood from each of three 2-week-old chicks of average weight was ob-

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