

A Study on the Possibility of Cystine Synthesis in the Rumen of Sheep together with the Effect of Cystine Supplementation on the Nitrogen Utilization of Lucerne in Young Stock.

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It has now been fully established in rats (1939) that lucerne is definitely deficient in the sulphur containing amino acids and that supplementation with either cystine or methionine enhances the biological utilization of lucerne nitrogen in a marked and highly significant manner. When, however, the effect of cystine on the nitrogen utilization of lucerne in mature sheep was investigated (1938) no beneficial effects could be established. It was therefore tentatively concluded that either lucerne contained enough cystine or methionine for maintenance, that cystine can be synthesized by the ruminal flora, or that cystine is not a necessary component for maintaining the nitrogenous integrity of the tissues in sheep. Burroughs and Mitchell (1940) have recently demonstrated the indispensability of cystine or methionine for endogenous purposes of rats in a convincing manner. Consequently our previous assumption of the dispensability of cystine for the maintenance of sheep falls away if the maintenance requirements of the two species are the same and hence the entire problem of cystine metabolism in sheep is reduced to a study of the quantitative cystine requirements and the probability of cystine synthesis in the rumen. Both these aspects have received a fair amount of attention in the past. It is nevertheless apparent that the past disputes on cystine synthesis and cystine requirements of sheep were almost exclusively based on the supposed gross differences between the cystine content of the feed or pasture and that of the fleece. To explain such a difference Rimington and Bekker (1932) suggested that cystine may be synthesized through a symbiotic action of the intestinal flora. Fraser and Fraser-Roberts (1932) on the other hand tried to account for the high cystine content of wool by assuming that the wool follicle possessed a special mechanism by which transformation into cystine can be effected. Both these theories are highly speculative and without any experimental evidence to support them. Although the work of Abdel-Salaam and Leong (1938) and Guerrant, Dutcher and Brown (1937)

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with rats and McElroy and Goss (1939) with sheep prove that certain members of the vitamin B complex can be manufactured by organisms in the colon of rats and by the microflora of the rumen of sheep, it does not follow that cystine can be synthesized in the same manner. If, however, cystine can be synthesized as postulated by the above theories, it will naturally imply that other essential and indispensable amino acids of probably less complex structure will be effected in the same way, since it is hardly possible to assign such highly selective and specialized powers to the micro flora of the rumen as a particulate selection of one single amino acid for synthesis from a number of equally important members of the same group of substances. Such conditions are obviously impossible and contrary to the generally accepted theory of protein metabolism in that it rejects the basic idea of dispensability and indispensability of amino acids for growth and maintenance in sheep. It may be argued on the other hand that synthesis of cystine finds preference because of its sulphur moiety, which is the only different element in its own and the constitution of the various other indispensable amino acids. Such a theory can be discharged on the basis of Daniels (1918) and Geilings (1917) work in which no effect of the addition of elementary sulphur on the growth of rats and mice on a cystine deficient ration could be established.

On the whole it would therefore appear, that a preferential synthesis of cystine by ruminal flora seems very improbable. Neither are there any indications that the special task of cystine formation should be allotted to a special portion of the wool fibre, namely the follicle. Such an assumption would indeed stretch the physiological possibilities of the body into a type of specialization which is difficult to associate with its other functions and powers in normal metabolism. A simpler and easier way of accounting for the supposed differences in the cystine content between pasture and wool, on which the above hypotheses are mainly based, is to agree with Woodman and Evans that in reality there is enough cystine in the pasture to account for the cystine content of the fleece and that differences in the chemical estimation of cystine may account for the calculated differences. It is furthermore of the utmost importance in this respect, as Burroughs and Mitchell (1940) have pointed out, that cystine or the indispensable amino acid needs may vary with the function which the animal is asked to fulfil as well as with different species of animals. Hence it is quite possible that while cystine is indispensable for complete nitrogen utilization during maintenance, it may not necessarily be needed in such large concentrations as for tissue synthesis. Burroughs and Mitchell (1940) state in this connection that 30 to 50 per cent. of the nitrogen required for maintenance can be supplied from nitrogenous sources other than indispensable amino acids. In reality, therefore, the requirements for true protein or indispensable amino acids for maintenance purposes are, comparatively speaking, small and make it, therefore, possible for the bulk of exogenous protein or amino acids to be utilized for the growth and synthesis of such tissues as wool and hair. In fact these findings of Burroughs and Mitchell (1940) explain why animals may be maintained on a basal ration generally regarded as too low in protein and why growth can be obtained on a maintenance ration supplemented by non-protein nitrogen. Instead of assuming as in the

case of cystine, that the microflora synthesize protein out of these compounds, it would be more correct on the basis of Burroughs and Mitchell's work to ascribe the maintenance and growth obtained under such conditions to a partial replacement of the basal protein, for growth purposes, which would otherwise be necessary to supply that portion of the endogenous nitrogen requirements equally well supplied by non-protein nitrogen. It may be equally possible in this connection that our previous non-response to cystine with mature sheep was due to the level of protein intake, which may have supplied enough cystine for the maintenance needs. For this reason the same type of experiment was performed on growing sheep, and the effect of cystine studied on the nitrogen utilization of lucerne. In conjunction with this experiment the ruminal ingesta of sheep fed on the same lucerne and collected 12 hours after the last feeding was examined biologically for a possible cystine synthesis. The biological value was determined on rats for ruminal ingesta, ruminal ingesta plus cystine, incubated lucerne and lucerne. By this means, it seemed possible to determine whether cystine is indispensable, supplements lucerne which is known to be deficient in cystine, or is synthesized in the rumen of sheep.

EXPERIMENTAL.

In the experiment on sheep, 5 merino wethers which were accustomed to the metabolism crates, were used. After a standard protein ration of lucerne, they were subjected several times to nitrogen low periods, during which the metabolic faecal nitrogen was determined. From these results an average value was obtained, which was utilized for the calculation of the metabolic faecal portion during the periods of lucerne feeding. The endogenous nitrogen was calculated from our equation $P = .74 W^{.734}$. The feeding periods were so arranged that a lucerne period was followed by a lucerne plus cystine followed again by a lucerne period. The lucerne intake was kept constant in each period, the only difference being the inclusion of cystine in the second period. The collection lasted 10 days, after which representative aliquots of the periods' collection of faeces and urine were taken and analysed for total nitrogen. In the second experiment four sets of six rats weighing approximately 70 grams each were put on lucerne, incubated lucerne, rumen ingesta and rumen ingesta plus cystine. Each set of rats was subjected to nitrogen low periods for the estimation of the metabolic faecal nitrogen and endogenous nitrogen. The rumen ingesta was obtained from sheep 12 hours after they were fed lucerne taken from the same stock as that on which the biological values were determined. The collection of the rumen ingesta was performed either by means of rumen fistula or by killing sheep after the stipulated period. The ingesta were dried at a low temperature, ground and mixed with the rest of the rations. Lucerne coming from the same batch as fed to the sheep was mixed with water and incubated at 37°C. for 24 hours. It was then washed and dried in the same manner as the rumen ingesta. This product constituted what is called incubated lucerne. The percentage composition of the rations is given in Table 1.

RESULTS.

In Table 2 are given the complete results on the metabolism studies of rats. Due to the exceedingly high concentration of ingesta in the ration at an 8 per cent. level it was decided, in order to maintain the palatability of the ration, to determine the biological value at a 6 per cent. protein level.

As will be seen from the data on lucerne an average biological value of 67 was obtained. This value as would be expected is slightly higher than that obtained previously by us at an 8 per cent. protein level. The apparent and true digestibilities are 50 and 75 per cent. respectively. There is only a slight and insignificant variation among the individual biological values, which may partly be attributed to the fact that the food intake was kept constant throughout the collection period.

In comparing the results obtained on lucerne with that on the incubated lucerne and rumen ingesta it will be noticed that no statistical difference is obtained in the results between the former and lucerne, while a much higher biological value is obtained for rumen ingesta of which the apparent and true digestibilities are lower than either lucerne or incubated lucerne. The biological values are 67 for lucerne, 64 for incubated lucerne and 82 for ruminal ingesta while the apparent and true digestibilities in the same order are 50 and 75, 48 and 73 and 40 and 66 per cent. respectively. On the basis of these figures it is clear that the difference in biological value between lucerne and rumen ingesta fed at the same level of nitrogen is by no means conclusive evidence that cystine synthesis was accomplished. In fact the higher level of absorbed nitrogen in the case of lucerne may in itself be responsible for a lowering of the biological value. Granted, however, that this difference in digestible protein intake cannot cause such a big difference in the respective biological values, then still it is quite possible that the microflora of the rumen may utilize the lucerne nitrogen for the synthesis and construction of their own tissues, until the cystine deficiency limits further utilization. These microflora now form a prominent part of the mixed ruminal ingesta protein, which is definitely more complete than lucerne. Hence there is a wide difference in the quality of the proteins contained in ruminal ingesta and lucerne as fed to our experimental rats. The difference which may account for the difference experienced in the biological values may be exclusively due to the presence of microflora, which in all probability have a better constituted protein complex than lucerne. Actually, therefore, the rôle of the microflora is purely symbiotic in nature, that is, they increase and exist on the exogenous supply of protein coming into the rumen. These microflora and fauna may as part of their life cycle become continually available to the host animal and consequently digested on similar lines as feed proteins. Whether the same policy of interception is practiced under all dietary conditions is difficult to say. It is quite possible that under conditions of nitrogen scarcity, such as under nitrogen free or nitrogen low feeding conditions these micro organisms may react quite differently. In this respect it is well to remember that Aberhalden and Rona (1905) have shown that bacteria and moulds

can synthesize protein from inorganic nitrogen and also that several investigators have obtained successful maintenance and growth on ruminants with urea feeding. The validity of the latter findings naturally depends, as was previously pointed out, to a large extent on the protein content of the basal ration. If the basal ration is protein free and urea addition produces growth or even supply the maintenance needs, it is apparent that either the microflora synthesize certain indispensable amino acids, or that the maintenance requirement of protein for rats as postulated by Burroughs and Mitchell is not applicable to ruminants.

The question of paramount importance is whether the microflora can synthesize their own tissue protein, which naturally requires a complete assortment of indispensable amino acids, from lucerne which is known to be deficient in cystine? Two possibilities suggest themselves. Either the microflora utilize the amino acids of lucerne until the sulphur amino acids limit further utilization; that is, a certain portion of the amino acids contained in lucerne whose utilization is limited by a cystine deficiency remains as part of the rumen ingesta and may from time to time or as a continual process pass through and be utilized by the sheep, or the microflora synthesize cystine at a rate comparable to the cystine deficiency in lucerne. In the latter case one would expect a complete protein mixture or at least one whose biological value is equivalent to lucerne supplemented by cystine that is 95. However, this is not the case since a biological value of only 82 is obtained for ruminal ingesta. If, on the other hand the first assumption is correct then one would expect a higher biological value than that of lucerne but not necessarily as high as that of lucerne supplemented by cystine, since the microflora which is now part of the ingesta can only construct protoplasm as long as the cystine in lucerne is available. The biological value will be increased in proportion to the amount of microflora to lucerne present. A certain portion of lucerne protein or its equivalent in amino acids is nevertheless retained in the rumen. The biological value of this portion will naturally be enhanced in the same manner as lucerne by the addition of cystine. This is what actually happens. Although the biological value of ruminal ingesta is 82 and hence better than lucerne, its value is further enhanced by the inclusion of 0.2 per cent. cystine, so that it ultimately attains a biological value of 95. It is interesting in this respect that if lucerne is supplemented by cystine its nitrogen becomes 28 per cent. better utilizable. When lucerne is fed to sheep, then the rumen ingesta collected 12 hours after the last feeding has a biological value of 82. If the ingesta are further supplemented by cystine, a biological value of 95 is obtained. Thus the biological value of lucerne after it has gone through the ruminal ingesta stage and supplemented by lucerne, is increased by 28 per cent., exactly the same as when it was supplemented by cystine directly. It would appear therefore as if the influence on the biological value of the microflora in the rumen is solely a question of an increased proportion of microflora in relation to lucerne protein and that no synthesis of cystine took place.

In regard to the sheep experiment the situation, on the basis of the rat experiments, becomes better understandable and easier to

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explain. The non-supplementary effect which we previously obtained when lucerne was fed to mature sheep is in all probability explained by the fact that lucerne has enough cystine to cover that portion of the maintenance requirement which require a complete assortment of indispensable amino acids. The rôle of the microflora in this respect is probably purely symbiotic, that is, taking the lucerne nitrogen, converting it into their own tissues and serving it back to the animal in that form. Burroughs and Mitchell have shown that the maintenance requirement of indispensable amino acids are indeed small and may furthermore vary with the species of animal as well as with the function it is asked to fulfil. Thus it can easily be appreciated, that even, with the shortage of cystine lucerne can still supply the maintenance needs of sheep. However, when growth is superimposed on maintenance the requirements of cystine become heavier and cystine definitely becomes a limiting factor in the nitrogen utilization of lucerne. Hence with the supplementation of cystine the biological value of lucerne is increased in young sheep by 10 per cent.

Both the experiments on rats and sheep afford no evidence of a cystine synthesis by the microflora of the rumen. In fact the evidence seems to indicate that the microflora follows a kind of interception policy, that is utilizing the exogenous nitrogen for its own purposes and rendering it available to the animal in micro organism form. From these observations it would appear as if the micro organisms play no intimate rôle in the protein metabolism of the ruminant. However, the latter assumption can only be substantiated by a careful study of the microflora population on nitrogen free diets, and the rôle they play when organic and inorganic nitrogen compounds are included in nitrogen free rations.

SUMMARY AND CONCLUSIONS.

By means of metabolism experiments on rats it was shown that the biological value of the rumen ingesta is better than that of lucerne. When rumen ingesta are supplemented by cystine their biological value is significantly enhanced. In sheep experiments it was shown that the inclusion of cystine in a lucerne ration for mature sheep did not influence the biological value of the latter. For growing sheep the supplementation of lucerne by cystine increased its biological value by 10 per cent. It is concluded that the action of the micro organisms is purely a question of interception, that is, utilizing exogenous protein for its own use and supplying it back to the animal in the bacterial and infusorial form.

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TABLE 1.

Composition of Rations on Percentage Basis.

	I.	II.	III.	IV.	V.
Lucerne.....	36.8	—	—	—	—
Ruminal Ingesta.....	—	41.1	39.7	—	—
Incubated lucerne.....	—	—	—	—	41.4
Dex. starch.....	37.2	32.9	34.1	70.0	32.6
Sucrose.....	10.0	10.0	10.0	10.0	10.0
Butter fat.....	8.0	8.0	8.0	8.0	8.0
Cod liver oil.....	2.0	2.0	2.0	2.0	2.0
Harris yeast.....	2.0	2.0	2.0	2.0	2.0
*Salt mixture.....	3.0	3.0	3.0	3.0	3.0
NaCl.....	1.0	1.0	1.0	1.0	1.0
Agar.....	—	—	—	2.0	—
Cystine.....	—	—	0.2	—	—
Egg white.....	—	—	—	2.0	—
Total.....	100.0	100.0	100.0	100.0	100.0
Percent nitrogen.....	1.12	1.13	1.03	0.47	1.10

* New salt mixture of Hubbel, R., Mendel, J.B., and Wakeman, A.J. (*J. Nutr.* 14, 273-285, 1937.)

TABLE 2.
Metabolism Data and Calculation of Biological Value.
N-Low Period for Lucerne Per Cent. N = 47.

Rat No.	Initial Weight.	Final Weight.	Average Weight.	Daily Food Intake.	Daily N Intake.	Daily N in Faeces.	Body N in Faeces.		Food N in Faeces.	Ab. sorbed N.	Daily N in Urine.	Body N in Urine.		Food N in Urine.	Retained N.	Biological Value.	Apparent Digest.	True Digest.
							Per gm. Food.	Per Day.				Per 100 gm.	Per Day.					
1.....	88	80	84	4.3	—	—	2.95	—	—	—	—	22.7	—	—	—	—	—	
2.....	90	81	86	6.3	—	—	2.78	—	—	—	—	20.0	—	—	—	—	—	
3.....	83	74	79	5.9	—	—	2.87	—	—	—	—	24.2	—	—	—	—	—	
4.....	85	77	81	5.1	—	—	2.93	—	—	—	—	26.8	—	—	—	—	—	
5.....	85	75	80	5.9	—	—	2.84	—	—	—	—	20.1	—	—	—	—	—	
6.....	93	86	90	5.4	—	—	2.88	—	—	—	—	—	—	—	—	—	—	

Lucerne Period Per Cent. N = 1.12.

1.....	83	81	82	6.0	67.2	35.0	2.95	17.7	17.3	49.9	32.9	22.7	18.6	14.3	35.6	71	49	74				
2.....	88	82	85	6.0	67.2	33.2	2.78	16.7	16.5	50.7	35.7	20.0	17.0	18.7	32.0	63	51	75				
3.....	76	74	75	6.0	67.2	30.5	2.87	17.2	13.3	53.9	38.0	28.5	21.4	16.6	37.3	69	55	80				
4.....	80	76	78	6.0	67.2	34.5	2.93	17.6	16.9	50.3	34.8	24.2	18.9	15.9	34.4	68	49	75				
5.....	80	76	78	6.0	67.2	36.7	2.84	17.0	19.7	47.5	36.6	26.8	20.9	15.7	31.8	67	45	71				
6.....	91	87	89	6.0	67.2	33.1	2.88	17.3	15.8	51.4	36.1	20.1	17.9	18.2	33.2	65	51	76				
															AVERAGE....	67	50	75				

N-Low Period for Ruminal Ingesta Per Cent. N = 47.

7.....	84	74	81	6.3	—	—	3.10	—	—	—	—	22.8	—	—	—	—	—	—
8.....	97	89	93	5.8	—	—	2.76	—	—	—	—	15.7	—	—	—	—	—	—
9.....	84	74	79	5.0	—	—	3.01	—	—	—	—	27.4	—	—	—	—	—	—
10.....	98	92	95	6.3	—	—	2.56	—	—	—	—	18.4	—	—	—	—	—	—
11.....	99	90	95	5.9	—	—	3.08	—	—	—	—	23.3	—	—	—	—	—	—
12.....	87	78	83	6.3	—	—	2.83	—	—	—	—	23.0	—	—	—	—	—	—

Ruminal Ingesta Period Per Cent. N = 1.13.

7.....	80	76	78	6.0	67.8	41.1	3.10	18.6	22.5	45.3	25.7	22.8	17.8	7.9	37.4	83	39	67				
8.....	89	85	87	6.0	67.8	39.1	2.76	16.6	22.5	45.3	24.3	15.7	13.7	10.6	34.7	78	42	67				
9.....	77	71	74	6.0	67.8	40.9	3.01	18.1	22.8	45.0	26.2	27.4	20.3	5.9	39.1	87	40	66				
10.....	97	89	93	6.0	67.8	40.7	2.56	15.4	23.3	42.5	22.5	18.4	17.1	5.4	37.1	87	40	63				
11.....	90	82	86	6.0	67.8	41.5	3.08	18.5	25.0	44.8	29.6	23.3	20.0	9.6	35.2	79	39	66				
12.....	78	74	76	6.0	67.8	40.4	2.83	17.0	23.4	44.4	25.9	23.0	17.7	8.2	36.2	81	40	65				
															AVERAGE....	82	40	66				

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TABLE 3.
The effect of Cystine Addition on the Biological Value of Lucerne in Young Sheep.
Lucerne Period.

Animal No.	Average Weight.	Food Consumption.		Dry Matter Intake.	N Intake.	N in Faeces.	Meta-bolic Faecal N.	Ab-sorbed N.	N in Urine.	Endo-genous N.	Food N Re-tained.	Bio-logical Value.	N Balance.	Ap-parent Digesti-bility.	True Digesti-bility.	
		Lucerne.	Starch.													
	Mgm.	Gm.	Gm.	Gm.	Gm.	Gm.	Gm.	Gm.	Gm.	Gm.	Gm.	Gm.	Gm.	%	%	
56408	30	360	200	515	10.04	4.29	2.06	7.81	4.25	1.44	5.00	64	1.54	57	78	
56417	23	360	200	515	10.04	4.48	2.06	7.62	3.64	1.16	5.14	67	1.92	55	76	
56436	23	360	200	515	10.04	4.40	2.06	7.70	3.49	1.16	5.37	70	2.15	56	77	
56386	23	360	200	515	10.04	4.19	2.06	7.91	4.17	1.16	4.90	62	1.68	58	79	
56387	24	360	200	515	10.04	4.52	2.06	7.58	3.84	1.22	4.96	65	1.58	55	75	
										AVERAGE.....		66		56	77	
<i>Lucerne plus Cystine (1 gram) Period.</i>																
56408	30	360	200	515	10.04	3.20	2.06	8.90	3.58	1.44	6.76	76	3.26	68	89	
56417	25	360	200	515	10.04	3.25	2.06	8.85	3.03	1.26	7.08	80	3.76	68	88	
56436	23	360	200	515	10.04	3.62	2.06	8.48	3.42	1.16	6.22	73	3.00	64	84	
56386	23	360	200	515	10.04	3.08	2.06	9.02	3.47	1.16	6.71	74	3.49	70	90	
56387	23	360	200	515	10.04	3.77	2.06	8.33	3.16	1.16	6.33	76	3.11	63	83	
										AVERAGE.....		76		67	87	
<i>Lucerne Period.</i>																
56408	30	360	200	515	10.04	3.13	2.06	8.97	4.39	1.44	6.02	68	1.52	68	89	
56417	25	360	200	515	10.04	3.58	2.06	8.52	3.95	1.26	5.83	68	2.61	65	85	
56436	23	360	200	515	10.04	3.70	2.06	8.40	3.80	1.16	5.76	69	2.54	63	84	
56386	23	360	200	515	10.04	4.21	2.06	7.89	4.20	1.16	4.85	61	1.63	58	79	
56387	23	360	200	515	10.04	4.22	2.06	7.88	3.81	1.16	5.23	66	2.11	58	79	
										AVERAGE.....		66		62	83	