THE EFFECT OF *dl*-METHIONINE, *l*-CYSTINE, AND *dl*-ISOLEUCINE ON THE UTILIZATION OF PARENTERALLY ADMINISTERED DOG HEMOGLOBIN

A Suggestion for Designing the Composition of the "Ideal" Protein Digest

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Recently we reported (11, 12) that dog hemoglobin given intraperitoneally in the form of laked red cells was well utilized by the protein-fasted dog to meet its protein requirements. In fact with a small supplement of *dl*-methionine, weight was well maintained and positive urinary N balance achieved. In contrast to reports (2, 4, 10) showing that human hemoglobin will not support growth in rats unless supplemented with isoleucine, *dl*-isoleucine supplementation alone was apparently without effect in the dog. When isoleucine was given intraperitoneally along with methionine and hemoglobin the N sparing effect was slightly greater than with methionine alone.

The present report extends our observations on the utilization of dog hemoglobin given intraperitoneally, particularly when supplemented with *dl*-methionine, *l*-cystine, and *dl*-isoleucine, singly and together. Specimens of dog hemoglobin and laked dog red cells have been analyzed for isoleucine by a microbiological method (7) and were found to contain 1.34 and 1.47 per cent isoleucine respectively[1] which are in good agreement with the 1.37 per cent isoleucine analysis of Brand and Grantham (3) done on the same specimen of dog hemoglobin. Brand has also reported that human and beef hemoglobins contain no isoleucine, and that dog hemoglobin contains 0.42 per cent and human hemoglobin 1.32 per cent methionine.

The experiments described below indicate that when hemoglobin administration is accompanied by oral supplementation with a combination of methionine and isoleucine, or methionine, cystine, and isoleucine, nitrogen utilization is significantly better than with the sulfur-containing amino acids alone. This implies that one cannot easily arrive at the best quantitative amino acid pattern for a given purpose by empirically altering one or more amino acids at a time.

Further efforts to demonstrate globin in the peripheral circulation after in-

[1] These analyses were kindly carried out by Robert H. Tully, 3rd, of the Department of Vital Economics.

jections of hemoglobin have yielded only negative results in experiments seeking to recombine any globin present with hemin at pH 8.5 in the presence of hydrosulfite. Invariably only strong plasma protein hemochromogen bands at 530 and 560 mμ were seen, and it is difficult to see how Fiessinger (6) was able to differentiate the hemoglobin band (at 556 mμ) in the presence of a strong hemochromogen band at 560 mμ in the serum of a patient with hemolytic jaundice. Chemical fibrinogen determinations were carried out on the plasma samples obtained during hemoglobin injection periods; the same plasma specimens were subjected to electrophoretic analysis and show that, for the most part, the increases in the β-globulin and "fibrinogen" peaks by electrophoretic analysis are attended by a closely corresponding rise in the chemically determined fibrinogen values. This rise in fibrinogen, and the low grade fever seen after many of the injections suggest low grade peritoneal irritation and perhaps pyrogens in the water used. The lack of a significant rise in α_2-globulin and the absence of an N catabolic effect which are seen almost invariably in acute inflammation in the dog, are against these animals having significant peritoneal inflammation.

Methods

The methods used in these experiments are identical with those previously described (11), except that the amino acid supplements were fed mixed with the basal non-protein diet instead of being injected as previously. The basal diet was fed at a level to supply about 75 calories per kilo body weight per day. The daily amino acid supplements[2] consisted of 0.34 gm. of *l*-cystine, 0.40 gm. of *dl*-methionine, and 1.0 gm. of *dl*-isoleucine, singly or together, as indicated in the individual experiments. Complete consumption of food and amino acid supplements was insured by forced feeding of any residue.

The N content of the urines, and the N content of the laked red cells used for the intraperitoneal injections were estimated by Kjeldahl. Blood fibrinogen levels were estimated by micro-Kjeldahl.

Rectal temperatures were determined daily at the same time immediately prior to the intraperitoneal injections. The temperatures in Tables 1 and 2 are those taken at the close of the periods indicated.

The electrophoresis studies of Table 3 were carried out as described in a former paper (1 3), in veronal buffer of pH 8.5 and ionic strength 0.1. In a special experiment 10 ml. of plasma obtained from dog 43–326 at the close of period 19 (Table 2) was diluted with an equal volume of veronal buffer of pH 8.5 and ionic strength 0.1 and dialyzed as usual against 2 liters of veronal buffer. After dialysis 10 ml. of the diluted plasma was mixed with 5 ml. of a freshly prepared solution of hemin containing 1 mg. of hemin per ml. The latter solution was prepared by dissolving crystalline hemin in a slight excess of 0.05 N sodium hydroxide and diluting to the proper volume with the veronal buffer of pH 8.5. The resulting solution of plasma and hemin was diluted to 20 ml. with the veronal buffer, reduced with a pinch of sodium hydrosulfite, and subjected to electrophoresis. After 3 hours the patterns were visualized by means of a cylindrical lens and inclined straight edge, and were then observed with a pocket spectroscope.

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[2] We are indebted to Merck & Company, Inc. for the amino acids used.

EXPERIMENTAL RESULTS

The experiments in Tables 1 and 2 were run concurrently and the results are mutually supporting. In Table 1 the excretion of urinary N rises gradually while hemoglobin intraperitoneally is the sole source of nitrogen (periods 4 through 8). This is essentially identical with basal hemoglobin periods previously described for this dog (9). The utilization of hemoglobin N is good, with maintenance of weight, and when compared with the periods 9 to 13, *l*-cystine produces no great obvious improvement in N retention. During these periods weight is held steady. Methionine supplementation on the contrary (periods 14 to 19) results in somewhat increased N retention and weight gain. The further addition of *dl*-isoleucine (periods 20 to 24) produces even greater improvement in N retention and weight gain. It thus appears that in the presence of methionine, a *dl*-isoleucine supplement results in even better N retention; yet, when methionine is withdrawn and the carry-over effect of methionine in period 25 is spent, isoleucine supplementation produces no better N utilization than with hemoglobin alone.

In Table 2 dog 43-326 shows good utilization of hemoglobin N with slight weight loss (periods 4 to 8); N utilization is not as good as that shown by dog 43-141. The addition of cystine results in some improvement in N retention, but slight weight loss continues. The further addition of methionine results in no better N utilization, but weight is regained slightly. The combined supplement of cystine, methionine, and isoleucine results in the best N utilization and continued weight gain. Here again the effects are not as large as seen in dog 43-141.

The withdrawal of the cystine and methionine results in a rapid increase of N excreted after the carry-over effect of methionine and cystine of period 25 is gone.

The more striking N utilization seen with the combined supplement of the amino acids than with either methionine or isoleucine alone in these experiments where the amino acids were fed is in contrast to the slight effect noted in a single experiment of our previous report (11) where the amino acid supplement was given *intraperitoneally*, and raises the question of the time of absorption of the amino acids as a factor in efficiency of utilization.

The rectal temperature changes noted in Tables 1 and 2 show no consistent variation that may be correlated with any of the changes in metabolism.

In Table 3 the increase in "ϕ" parallels the increase in chemically determined fibrinogen until after periods 14 to 19, when the peak is much too large. The significance of this increase is not known. At no stage in the experiment was there any increase in α_3-globulin. This argues against the existence of significant peritoneal irritation or tissue damage.

The following observations were made in the electrophoretic study of the reduced hemin and plasma of dog 43-326:—

TABLE 1

Laked Red Cells Intraperitoneally

Nitrogen Retention Improved by S-Containing Amino Acids Alone and with dl-Isoleucine

Dog 43-141-mongrel female

Period	Hemoglobin injected total N	Total urinary N	Urea N + NH_3-N	Weight	Rectal temperature
48 hrs.	*gm.*	*gm.*	*per cent*	*kg.*	
Basal diet contains little protein (0.52 gm., N per period)					
1		1.96	78.2		
2		1.51	62.0	10.3	
3		1.84	66.9		
Basal diet + hemoglobin intraperitoneally					
4	3.68	2.55	74.7	10.1	
5	3.83	2.62	72.6		
6	3.35	2.32	72.0		38.5
7	3.41	3.07	63.9		38.3
8	3.71	3.59	70.5	10.5	39.3
Total	17.98 (20.58)*	14.15			
Basal diet + hemoglobin + *l*-cystine					
9	3.51	3.09	69.4	10.4	38.4
10	3.49	2.67	63.0	10.5	38.6
11	3.46	2.44	65.5	10.5	39.3
12	3.26	3.30	73.0	10.5	40.0
13	2.61	2.54	66.0	10.5	38.4
Total	16.33 (19.31)*	14.04			
Basal diet + hemoglobin + *dl*-methionine					
14‡	3.14	2.05	64.2	10.6	39.0
15	3.07	2.83	69.0	10.7	39.0
16	3.39	2.29	69.2	10.7	39.0
17	3.50	2.81	75.6	10.8	38.9
18	3.57	2.68	69.3		38.4
19	3.72	2.40	71.4	10.9	38.8
Total	20.39 (23.37)*	15.06			
Basal diet + hemoglobin + *dl*-methionine + *dl*-isoleucine					
20	3.10	2.42	66.1	10.9	38.7
21	3.29	1.87	55.3	11.0	38.6
22	3.14	1.82	47.8	11.0	39.0
23	2.92	1.97	46.9	11.2	39.0
24	3.27	2.01	46.7	11.2	29.2
Total	15.72 (19.78)*	10.09			

TABLE 1—*Concluded*

Period	Hemoglobin injected total N	Total urinary N	Urea N + NH_3-N	Weight	Rectal temperature
48 hrs.	*gm.*	*gm.*	*per cent*	*kg.*	
Basal diet + *dl*-isoleucine					
25	3.23	2.04	57.2	11.1	38.1
26	3.33	3.55	73.6	11.1	38.9
27	3.48	3.19	69.8	11.1	38.6
28	2.92	3.36	73.4	11.1	38.4
29	3.29	3.13	71.7	11.1	
Total	16.25 (19.82)*	15.27			
Basal diet					
30		2.03	69.5		
31		1.96	61.2		
32		1.82	62.7		

* Totals in parentheses include the N of basal diet and amino acid supplements.

‡ Fecal N periods 14 through 20 averaged 0.64 gm. N per period.

Descending limb. The α_1-globulin peak was absent being apparently merged with the albumin. Reading from above downward, below the level of the β-peak the solution was brown due to the hemin. At the center of the rather rounded peak which represented α_2-globulin, the typical absorption bands of a hemochromogen appeared. Proceeding downward the bands became broader and more intense, finally merging at the level of the albumin at which point the solution became deep red. *Ascending limb.* From the level of α_1-globulin downward, there was complete light absorption, extending from the orange edge of the red through the violet. The solution was deep red throughout this region.

DISCUSSION

These observations confirm our previous impression of the efficiency with which the protein-fasted dog can utilize the N of parenterally administered dog hemoglobin, and emphasize the fact that not only the hemoglobin iron but also the protein N is avidly retained and reutilized to meet various body protein requirements. The degree of reutilization is conditioned by the low S and isoleucine contents of dog hemoglobin. Our previous experiments indicate that the isoleucine content of dog hemoglobin is enough for maintenance in some adult dogs while the virtual absence of isoleucine from human globin or beef globin (3) makes them incomplete proteins for growth in the rat, in the classical sense. The failure of an oral supplement of isoleucine alone to improve the utilization of dog hemoglobin where hemoglobin alone suffices for maintenance, indicates that the low S content (and low methionine content) of dog hemoglobin is a more primary limiting factor in this case. In these

TABLE 2

Laked Red Cells Intraperitoneally

Nitrogen Retention Improved by l-Cystine, dl-Methionine, Alone and When Mixed with dl-Isoleucine, But not by Isoleucine Alone

Dog 43-326-mongrel female

Period	Hemoglobin injected total N	Total urinary N	Urea N + NH_3-N	Weight	Rectal temperature
48 hrs.	*gm.*	*gm.*	*per cent*	*kg.*	
Basal diet. (0.54 gm. N per period)					
1		2.55	78.2		
2		2.35	68.7	12.7	
3		2.75	68.6		
Basal diet + hemoglobin intraperitoneally					
4	3.58	3.35	73.9	12.7	
5	4.29	3.53	72.8		
6	3.70	3.84	78.3		39.3
7	3.39	3.38	71.7		39.1
8	3.71	4.65	65.2	12.6	38.7
Total	18.67 (21.20)*	18.75			
Basal diet + hemoglobin + *l*-cystine					
9	3.69	3.24	72.6		
10	3.45	2.91	66.1	12.5	38.6
11	3.41	2.62	75.6		38.8
12	3.43	4.08	73.0		39.3
13	3.09	2.26	67.7	12.3	39.3
Total	17.05 (20.00)*	15.01			
Basal diet + hemoglobin + *l*-cystine + *dl*-methionine					
14‡	3.14	3.00	65.1	12.4	38.8
15	3.07	2.98	78.9		39.1
16	3.39	3.32	76.2		38.8
17	3.50	3.04	83.7		38.4
18	3.57	3.40	77.4		38.4
19	3.72	3.51	79.2	12.5	38.6
Total	20.39 (23.80)*	19.25			
Basal diet + hemoglobin + *l*-cystine + *dl*-methionine + *dl*-isoleucine					
20	3.10	2.92	77.6	12.5	38.9
21	3.29	2.34	55.6		38.8
22	3.14	2.04	—		38.6
23	2.92	1.99	55.2		38.6
24	3.27	2.73	65.7	12.7	38.8
Total	15.71 (20.22)*	12.02			

TABLE 2—*Concluded*

Period	Hemoglobin injected total N	Total urinary N	Urea N + NH_3-N	Weight	Rectal temperature
48 hrs.	*gm.*	*gm.*	*per cent*	*kg.*	
Basal diet + hemoglobin + *dl*-isoleucine					
25	3.23	1.92	62.8	12.8	38.9
26	3.33	4.82	79.1	12.7	40.5
27	3.48	4.28	75.6	12.6	39.3
28	2.92	3.85	76.2		39.7
29	3.29	3.38	72.6	12.6	
Total	16.24 (19.90)*	18.25			
Basal diet					
30		2.53	75.7	12.5	
31		1.69	58.4		
32		1.84	52.4		

* Totals in parentheses include the N of basal diet and amino acid supplements.

‡ Fecal N of periods 14 through 20 averaged 0.49 gm. N per period.

experiments a combined supplement of the S-containing amino acids and isoleucine at the levels used, results in even better N retention than with cystine or methionine alone. This points to the difficulty and futility of attempting empirically to arrive at the quantitative composition of an "ideal" amino acid mixture. For example, if one wished only to study the nutritional value of a mixture of ten amino acids, and limited the study to the effect on growth of mixtures containing each amino acid at one of three possible levels, then 3^{10} (or 59049) amino acid mixtures would have to be studied. And it is impossible to predict *a priori* what change in value will result from altering the level of a single amino acid or several at once.

Furthermore, the unpredictable differences in the quantitative amino acid requirements of different mammalian species in various stages of growth and development make the transfer of quantitative data on amino acid requirements from animals (such as the dog or rat) to man extremely hazardous. Hegsted, Hay, and Stare have presented data (8) which imply that the tryptophane requirement of the growing rat is 50 times that for maintenance in the dog, and the requirement for isoleucine in the rat 60 times that for maintenance in the dog (on a weight basis).

One approach to the problem of designing quantitatively the ideal amino acid pattern is opened up by the newer methods for amino acid analysis. It is now practically feasible to determine much of the amino acid composition of the total organism, be it rat, dog, or man in the various stages of development,

growth, and maturity. With these species-total organism patterns as prototypes, the known composition of dietary proteins or digests can be altered by adding those amino acids which vary considerably in amount from the amino acid pattern of the total organism. The "ideal" mixture may then be defined

TABLE 3

Intraperitoneal Hemoglobin Supports Electrophoretic Albumin Level and Increases Concentration of Proteins Migrating with Mobility of Fibrinogen

Dog 43-826

Period 48 hrs.	Total Protein	Albumin	α^+	β	ϕ°	γ	
Basal diet							
1–3	5.80	1.92	2.09	0.38	0.71 (0.19)*	0.70	
Basal diet + hemoglobin intraperitoneally							
4–8	5.47	1.81	1.46	0.47	0.92 0.45*	0.78	Slight rise in temperature
Basal diet + *l*-cystine + hemoglobin intraperitoneally							
9–13	5.16	2.14	1.03	0.36	0.94 0.38*	0.69	Slight rise in temperature
Basal diet + *l*-cystine + *dl*-methionine + hemoglobin							
14–19	5.97	2.05	1.59	0.34	1.32 0.42*	0.67	Normal temperature. α_1-globulin much increased
Same regimen for 22 days followed by basal diet for 8 days							
1 month later	5.08	1.77	1.03	0.21	1.34	0.70	α_1-globulin normal

α refers to the sum of the concentrations of all 4 α-globulins.

$^\circ$ only about 40 per cent of the normal ϕ peak is due to fibrinogen.

* Figures in parentheses represent plasma fibrinogen levels in grams per cent as determined chemically.

as having a composition identical with that of the total organism which is to be grown or maintained.

On this basis it appears reasonable, albeit teleologically, that the egg which is itself capable of giving rise to the total organism of the chick, is also the source of a protein mixture which has one of the highest known biological values. Likewise the whole mixed proteins of milk provide virtually the sole source of protein for the very rapid growth of infant mammals, and are by that very token of very high biological value.

Further refinements of the "ideal" amino acid pattern might be based on precise amino acid analyses of the separate organs of the growing or mature animals, and on estimates of the total turnover rates, or loss under conditions of greatest demand, as in fasting. The data of Addis (1) could be used for such approximate calculations for the rat. In spite of its obvious limitations such an "ideal" mixture it is felt would come closer to the theoretical optimum than a mixture arrived at empirically through more prolonged and arduous labors.

The rise in blood fibrinogen and the temperature elevations noted during hemoglobin injection periods are undoubtedly related to low grade peritoneal irritation and perhaps to pyrogens in the ordinary sterile distilled water used in preparing the laked red cells. The absence of an increase in the α_s-globulin peak and the lack of a protein catabolic response are two points against there being acute inflammatory reaction or tissue damage incident to the hemoglobin injections. The increase in the chemically determined blood fibrinogen is roughly equal to the equivalent increase in the areas of the β-globulin and "fibrinogen" peaks of the electrophoretic patterns. This is against the previous hypothetical assumption that circulating globin might be the cause of the rise in the β-globulin fibrinogen peaks.

In the special electrophoretic study of reduced hemin in the plasma of dog 43-326 it was found that virtually all of the pigment moved along with the α_1-globulin and albumin. Spectroscopic examination of the red pigmented zones showed absorption typical of hemochromogens. This is of interest when compared with Fairley's observations on the reaction of hemin with only the albumin fraction of human or simian plasma (5). If any globin were present in this specimen of plasma and were converted to hemoglobin by the hydrosulfite reduction, it would certainly be obscured by the dense hemochromogen absorption in the pigmented zones. If globin or some closely related protein (other than hemoglobin) occurs in the peripheral circulation after hemolysis or massive hemoglobin infusions, some more highly specific method such as an immunologic procedure will have to be developed to detect it.

SUMMARY

1. Further observations on the utilization of parenterally administered dog hemoglobin show that oral supplements of *dl*-methionine and *l*-cystine improve the efficiency of utilization of hemoglobin N, while a fed supplement of *dl*-isoleucine alone is without effect.

2. When *dl*-isoleucine is added to a fed supplement of methionine or methionine and cystine, the utilization of parenterally given hemoglobin N is even better than with the sulfur-containing amino acids alone.

3. A suggested approach to the problem of designing the quantitatively "ideal" amino acid mixture lies in the definition of what may be called total organism–amino acid patterns of rat, dog, man, etc. These may vary con-

siderably not only at different developmental stages in a given species, but also certainly from one species to another.

4. Further attempts to detect globin in the peripheral circulation have pointed to the need for a highly specific procedure such as that an immunologic method may offer.

5. Reduced hemin in dog plasma migrates with α_1-globulin and albumin in veronal buffer at pH 8.5 and the colored zones give strong hemochromogen absorption bands.

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