LXIV. CASEINOGEN FOR VITAMIN TESTS.

BY KATHARINE HOPE COWARD,
KATHLEEN MARY KEY,
FREDERICK JOHN DYER
AND BARBARA GWYNNETH EMILY MORGAN.

*From the Pharmacological Laboratories, Pharmaceutical Society
of Great Britain.*

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THE basal diet used in vitamin A testing should, when supplemented by cod-liver oil, allow growth to maturity at what may be considered a normal rate; and the mean growth response of groups of animals should be graded to the dose of cod-liver oil given. If growth is restricted by the lack of some factor other than vitamin A, the result, at the best, may show such small differences in response to different doses of cod-liver oil as to make the significance of the differences doubtful. At the worst, the results may be so very irregular that no conclusion whatever can be drawn from them. It has recently been shown [Coward, Key, Dyer and Morgan, 1930] that the kind of caseinogen used in the basal diet for vitamin A testing is of the greatest importance in this respect. One sample of "vitamin-free casein," failed completely to fulfil the required conditions. Some of the rats grew well but the mean responses of groups of rats to different doses of cod-liver oil were slightly graded to the lower doses only and, at a comparatively low level, reached a limit which was far below any "normal" rate. The caseinogen was suspected of being the limiting factor and a fresh sample was used. "Light white casein" bought from the British Drug Houses Ltd. was extracted cold once with alcohol and four times with ether (24 hours each extraction), and the whole experiment repeated with satisfactory results. On the higher doses of cod-liver oil, the growth response of the groups might be considered almost "normal." It was probably as great as could ever be obtained immediately after the cessation of growth due to a deficiency of vitamin A. Moreover, the differences in response of different groups of rats to doses differing by 100 % were great enough to be considered significant even when the possible error of the estimation was allowed at 2ϵ where $\epsilon = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$. This held through a range of doses from 0.25 mg. to 7.5 mg.

The extraction of the caseinogen was, however, both costly and laborious and it occurred to us that the greater growth-promoting power of "light white casein" formerly ascribed to the presence in it of vitamin A might, in

reality, be due to whatever constituted the difference between it and the "vitamin-free casein" which we have described in recent papers. We accordingly made comparisons in different ways between "light white casein" extracted as we were in the habit of extracting it and "light white casein" not treated in any way. We also compared untreated "light white casein" with some of the same batch heated in exactly the same way as Messrs Glaxo heat their caseinogen to make the "vitamin-free casein." We are satisfied that the extraction of the "light-white casein" is entirely unnecessary. It follows that the heating process of Messrs Glaxo is therefore unnecessary and moreover we have shown that it is slightly but definitely harmful to the caseinogen.

METHOD.

1. *Exp. 1. Comparison of caseinogens as the protein of a vitamin A-free diet.*

A large batch (2 cwts.) of "light white casein" (B.D.H.) was thoroughly mixed. A part of it was left untreated, a second part of it was extracted once with alcohol, 4 times with ether, cold (24 hours each extraction) and a third part was, through the courtesy of Messrs Glaxo, heated in thin layers as they heat their own caseinogen to render it "vitamin-free." The three parts are here referred to as

- (a) untreated "light white casein";
- (b) extracted "light white casein";
- (c) treated "light white casein."

The three forms of caseinogen were incorporated in vitamin A-free diets in the usual way (*i.e.* 15 % caseinogen, 73 % dextrinised rice starch, 8 % dried yeast and 4 % (Steenbock's 40) salt mixture; the diet was given to the rats *ad lib.* together with about 2 units vitamin D per rat twice a week). The three diets were tested on large groups of animals to determine (a) the average length of time required for the animals to cease growing, (b) the average weight at which the animals ceased growing. Table I gives a summary of the results of this experiment. Details are given in Tables II, III and IV. It is evident that (a) the times taken for groups of animals to cease growing and (b) the average weights at which these animals cease growing are the same

Table I. *Summary of results of Exp. 1.*

To compare the growth of groups of rats given a vitamin A-free diet containing

- (a) untreated "light white casein" (B.D.H.),
- (b) extracted "light white casein" (B.D.H.) (once alcohol, 4 times ether),
- (c) treated "light white casein" (B.D.H.) (heated in thin layers).

		Average of highest weights attained g.	Average weights at which considered steady g.	Average of times taken for weights to become steady (days)
(a) Untreated L.W.C.	±0.4	110.0	102.8	41.9
		105.5	99.7	53.8
(b) Extracted L.W.C.	±0.4	110.7	102.8	38.9
		101.6	96.1	43.8
(c) Treated L.W.C.	±0.4	82.1	77.1	34.2
		81.8	77.1	38.5

whether untreated or extracted "light white casein" is used in the basal diet. On the other hand, the animals whose basal diet contained the treated caseinogen ceased growing in a shorter time and at a lower weight than either of the other two groups. Two interpretations of this are possible. Either the "light white

Table II.

To show: (a) the average time taken by a group of rats to become steady in weight on a vitamin A-free diet containing untreated "light white casein."
 (b) the average weight at which these rats became steady.
 (c) the effect of giving 5 of these rats a daily dose of 0.1 g. of a vitamin margarine for 5 weeks.

"Light white casein," untreated in the vitamin A-free diet.

	Rat	Initial weight	Highest weight attained	Weight when considered steady	Time taken to become steady (days)	Dose	Response in 35 days
		g.	g.	g.			g.
♂	M 4804	50	133	132	45	—	—
♂	M 4853	43	139	136	59	—	—
♂	M 4852	49	139	128	50	—	—
♂	M 4898	45	132	109	49	—	—
♂	M 4900	47	125	114	42	—	—
♂	M 4856	46	113	112	45	—	—
♂	M 4855	47	137	117	48	—	—
♂	M 4899	41	93	91	45	—	—
♂	L 2	48	170	162	78	—	—
♂	2323	38	105	98	36	—	—
♂	M 4896	47	109	97	37	—	—
♂	M 4854	42	72	70	24	0.1 g. G. Ch. marg.	43
♂	M 4810	44	82	72	24	0.1 g. G. Ch. marg.	48
♂	2342	46	54	52	22	0.1 g. G. Ch. marg.	69
♂	M 4809	42	109	105	42	—	—
♂	M 4806	46	85	84	42	—	—
♂	M 4897	46	163	155	63	—	—
♂	M 4851	44	53	46	20	0.1 g. G. Ch. marg.	— 3, dead* in 2 days
♂	M 4811	46	77	73	24	0.1 g. G. Ch. marg.	42
♂	M 4895	(56)	(82)	(68*)	(42)	—	—
		Av. = 45.1	Av. = 110.0	Av. = 102.8	Av. = 41.9		
♀	M 4864	49	110	107	56	—	—
♀	M 4803	44	109	90	66	—	—
♀	M 4865	44	124	120	67	—	—
♀	L 5	44	120	115	77	—	—
♀	M 4868	45	101	97	46	—	—
♀	M 4867	(47)	(125)	Discarded	(76)	—	—
♀	M 4804	41	111	103	42	—	—
♀	M 4802	42	105	104	50	—	—
♀	M 4807	42	93	91	42	—	—
♀	M 4808	45	85	80	42	0.1 g. G. Ch. marg.	16
♀	M 4805	40	97	90	50	—	—
♀	M 4866	(44)	—	(38*)	(18)	—	—
♀	L 6	(36)	—	(36*)	(13)	—	—
		Av. = 43.6	Av. = 105.5	Av. = 99.7	Av. = 53.8		
							Av. + 43.6 (omitting*)

* Rat died, not included in averages.

casein" contains vitamin A which has not been removed by extraction but has been destroyed by heat, or the "light white casein" does not contain any vitamin A and the heat has damaged it as protein or some unknown factor in it. The later experiments throw light on this matter.

2. *Exp. 2. The result of substituting untreated "light white casein" for (a) extracted "light white casein" or (b) treated "light white casein" in the vitamin A-free diets of rats which have ceased to grow on these diets.*

(a) Fifteen rats (6 bucks and 9 does) which had become steady in weight on a vitamin A-free diet containing extracted "light white casein" were given a diet containing untreated "light white casein," to see whether the amount

Table III.

To show: (a) the average time taken by a group of rats to become steady in weight on a vitamin A-free diet containing extracted "light white casein";
(b) the average weight at which these rats became steady;
(c) the effect of substituting untreated "light white casein" for extracted "light white casein" in this diet.

"Light white casein" in a vitamin A-free diet.							
Rat	Extracted				Untreated		
	Initial weight g.	Highest weight attained g.	Weight when considered steady g.	Time taken to become steady (days)	Increase if any on this "casein" g.	Total response g.	Time taken for this response (days)
♂ M 4876	52	94	85	41	1	-9	13
♂ M 4904	50	129	117	38	1	-16	12
♂ M 4873	51	111	108	40	—	-20	16
♂ M 4875	47	112	103	40	—	-36	13
♂ M 4906	55	126	122	38	—	-10*	18
♂ 2325	38	92	82	36	—	-10*	2
♂ M 4902	(46)	(126)	(104*)	(35)	—	—	—
♂ M 4903	(50)	(101)	(84*)	(24)	—	—	—
♂ M 4874	(43)	(43)	(32*)	(28)	—	—	—
♂ M 4905	(49)	(49)	(39)	(15)	Died within 6 days of being put on a test; therefore discarded Found wandering in rat room; therefore discarded		
♂ 2344	(43)	(44)	(40)	(18)			
♂ M 4901	(40)	(40)	(34*)	(14)	—	—	—
Av. = 48.8 Av. = 110.7 Av. = 102.8 Av. = 38.9							
♀ M 4862	48	128	125	43	5	-11	30
♀ M 4870	46	91	89	46	14	13	24
♀ M 4871	51	85	82	40	—	-8	21
♀ M 4861	52	125	117	38	—	-17	21
♀ M 4863	42	94	82	49	3	-10	14
♀ M 4860	45	90	84	43	—	-18	9
♀ M 4872	49	85	81	46	—	-26*	7
♀ M 4869	50	88	82	40	—	-22*	15
♀ M 4858	45	128	123	49	7	-13	31
♀ M 4857	(47)	(47)	(36*)	(17)	—	—	—
Av. = 47.5 Av. = 101.6 Av. = 96.1 Av. = 43.8							

* Rat died, not included in averages.

of vitamin which might be present in the untreated sample would be sufficient to make the rats resume growth. The result has been summarised in Table III. It may be seen that 4 rats have increased in weight (5, 14, 3, 7 g. respectively), 2 have gained 1 g. which is not considered significant, 9 have lost weight. It is evident that the amount of vitamin A, if any, in untreated "light white casein" is negligible.

(b) Nineteen rats (7 bucks and 12 does) which had become steady in

weight on a vitamin A-free diet containing treated "light white casein" were given a diet containing untreated "light white casein." The response to this change was greater than the response when extracted was changed to untreated "light white casein." Seven rats increased in weight (11, 9, 14, 19, 16, 2, 5 g. respectively) and the rest lost weight or gained only 1 g.

Table IV.

To show (a) the average time taken by a group of rats to become steady in weight on a vitamin A-free diet containing treated "light white casein";
 (b) the average weight at which these rats became steady;
 (c) the effect of substituting untreated for treated "light white casein" in the diet;
 (d) the effect of giving 5 of these rats a daily dose of 0.1 g. of a vitamin margarine for 5 weeks.

Rat	Treated					Untreated		
	Initial weight g.	Highest weight attained g.	Weight when considered steady g.	Time taken to become steady (days)	Increase if any on this "casein" g.	Total response g.	Time taken for this response (days)	Response in 35 days g.
♂ M 4879	44	85	82	34	11	+10	16	—
♂ M 4800	44	100	95	43	—	-17	16	—
♂ M 4798	47	92	79	34	1	-7	25	—
♂ M 4878	51	60	57	32	—	-5	17	—
♂ L 3	39	80	75	50	9	+5	16	—
♂ 2324	38	64	64	36	—	-12*	8	—
♂ M 4797	49	100	97	42	—	-30*	18	—
♂ L 1	49	61	56	24	—	—	—	—
♂ M 4801	43	90	80	28	—	—	—	0.1 g. G. Ch. marg. 27
♂ M 4799	49	100	96	29	—	—	—	0.1 g. G. Ch. marg. 9
♂ M 4796	48	71	67	24	—	—	—	0.1 g. G. Ch. marg. 24
♂ M 4880	(53)	(112)	(102*)	(32)	—	—	—	0.1 g. G. Ch. marg. 35
♂ M 4877	(50)	(111)	(105*)	(32)	—	—	—	—
Av. = 45.5 Av. = 82.1 Av. = 77.1 Av. = 34.2								
♀ M 4888	44	74	69	31	14	+1	21	—
♀ M 4850	51	111	108	36	—	-5	16	—
♀ M 4846	44	94	88	43	—	-9	27	—
♀ M 4891	50	93	86	43	19	-1	23	—
♀ M 4849	52	112	110	45	16	+5	28	—
♀ M 4848	49	85	82	43	—	-10	13	—
♀ L 4	37	64	62	45	—	-12*	11	—
♀ M 4890	42	59	50	24	—	-5*	3	—
♀ 2343	44	68	67	39	2	-12*	12	—
♀ M 4887	45	77	74	31	—	-2*	14	—
♀ M 4845	47	76	76	38	5	-11*	11	—
♀ M 4844	48	48	45	17	—	—	—	0.1 g. G. Ch. marg. -1
♀ M 4892	47	102	95	66	—	-10	3	—
♀ M 4893	(43)	(78)	(56*)	(24)	—	—	—	—
♀ M 4847	(49)	(102)	(86*)	(49)	—	—	—	—
♀ M 4889	(53)	(68)	(53*)	(24)	—	—	—	—

Av. = 40.2 Av. = 81.8 Av. = 77.1 Av. = 38.5

* Rat died, not included in averages.

It is concluded that there is less difference between the extracted and untreated caseinogen than between the treated and untreated. Indeed the difference between the extracted and untreated is considered insignificant.

3. *Exp. 3. The effect of giving equal doses (0.1 g.) of a vitamin margarine to rats which had ceased to grow on a vitamin A-free diet containing (a) untreated "light white casein" and (b) treated "light white casein."*

Four bucks and one doe were used in each part of this experiment. (A fifth buck in one group died after 3 days and was therefore discarded.) The result is summarised in Table V. Details are given in Tables II and IV.

This result is open to the interpretation that the small amount of vitamin A which might be present in the untreated "light white casein," although not enough to prevent cessation of growth, might supplement the vitamin A of the vitamin margarine and thus cause the greater growth of this group of animals. This explanation, however, seems to be impossible in view of the work described in earlier papers, for we have found that even when excessive amounts of vitamin A in the form of 0.2 g. cod-liver oil daily have been given, we have obtained remarkable increases in growth rates on substituting "light white casein" (B.D.H.) for the "vitamin-free casein" (Glaxo). It seems much more probable that the treated caseinogen has been altered in some way so that it is less adequate for growth than the untreated form.

Table V.

Caseinogen	Dose of vitamin margarine	Average increase in weight of the group in 5 weeks
Untreated	g. 0.1	g. 43.6
Treated	0.1	18.8

4. *Exp. 4. The effect of giving cod-liver oil to rats which have become steady in weight on a vitamin A-free diet containing (a) untreated "light white casein" and (b) extracted "light white casein."*

Where the same dose of cod-liver oil has been used for comparing the two forms of caseinogen, results are, of course, directly comparable. Where different doses have been used, the results are compared by reference to a curve relating response to dose of oil. The curve which we have worked out for our own laboratory has already been described [Coward, Key, Dyer and Morgan, 1930]. The equation for this curve is $y = 12.1 + 40.24 \log x$, where y = the mean increase of a group of animals all given the same dose, and x = the dose of cod-liver oil in mg. It has been found that this curve can be resolved into two somewhat similar curves, one for the bucks used in its construction and one for the does. These curves are:

$$y = 11.3 + 50.3 \log x \text{ (bucks).}$$

and

$$y = 12.4 + 27.4 \log x \text{ (does).}$$

When it has not been possible to use equal numbers of bucks and does for a test, comparisons may be made by means of the two curves. The average of the results will then be more accurate than a direct comparison made through the original curve. We now express the vitamin A potency of any sample examined in our laboratory as a percentage of the potency of the oil which we used in the making of these curves and which we have temporarily adopted as a standard. We find the mean increase of a group of rats (about 10) given a certain dose of cod-liver oil daily for 5 weeks, then find by the curves what dose of our standard oil would have given that increase and thus determine the relative potency of the oils. In this way we found two oils, B and C, to have the same vitamin A potency relative to oil A whether they were tested on a diet containing untreated "light white casein" or on one containing extracted "light

white casein." Table VI gives a summary of these figures and Table VII gives the details.

This result is regarded as additional evidence that the amount of vitamin A in "light white casein," if any, is negligible, and that the laborious and expensive process of extracting "light white casein" is superfluous.

Table VI.

Oil	Potency expressed as a percentage of the potency of oil A when examined on basal diets containing	
	(a) Untreated caseinogen	(b) Extracted caseinogen
B	83.05	72.9
C	173.8	189.6

Table VII.

To show that the vitamin A potency of an oil relative to another oil is the same whether determined with untreated "light white casein" or with extracted "light white casein" in the basal diet.

Oil	Dose mg.	No. of bucks	Average increase in 5 weeks g.	Dose of oil A which brought about this increase mg.	No. of does	Average increase in 5 weeks g.	Dose of oil A which brought about this increase mg.	Relation to oil A (%) according to	
								Bucks	Does
B	1.0	10	4.2	0.723	9	11.8	0.95	72.3	95
	untreated caseinogen							Average Weighted mean = 83.65 = 83.05	
	4.0	4	35.75	3.06	5	24.6	2.79	76.5	69.7
	extracted caseinogen							Average Weighted mean = 73.1 = 72.9	
C	1.0	6	27.7	2.118	13	20.2	1.9275	211.8	192.75
	untreated caseinogen								
	5.0	7	54.9	7.357	11	36.2	7.393	147.1	147.9
	untreated caseinogen							Average Weighted mean = 174.9 = 173.8	
	5.0	13	61.4	9.91	4	37.25	8.075	198.2	161.5
	extracted caseinogen							Average Weighted mean = 179.85 = 189.6	

SUMMARY.

It has been shown in several ways that "light white casein" (B.D.H.) may be safely used without extraction for vitamin A tests.

1. Large groups of rats were given a vitamin A-free diet containing (a) extracted or (b) non-extracted "light white casein" (B.D.H.). They became steady in weight at about the same weight and in about the same length of time.

2. Rats which had become steady in weight on a vitamin A-free diet containing extracted "light white casein" were given an exactly similar diet

but containing non-extracted "light white casein"; they did not resume growth but continued to lose weight.

3. The estimation of the vitamin A potency of a sample of cod-liver oil relative to another sample used as a standard of reference in these laboratories was the same whether extracted or non-extracted "light white casein" was used in the basal diet.

The extraction of the caseinogen has not removed the growth-factor whose existence has recently been demonstrated by Coward *et al.* [1929].

The heating of "light white casein" by Messrs Glaxo in the way in which they prepare their "vitamin-free casein" only partially destroyed this factor.

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APPENDIX.

The method of determining the equations of the curves.

The mean increases (y) of the groups of rats given different doses of cod-liver oil for a period of 5 weeks were plotted against the logarithms of the doses of oil given. They fell practically on a straight line. The best straight

Fig. 1. Curves relating mean increases in weight in 5 weeks of groups of rats (bucks and does respectively) to the logarithms of the doses of cod-liver oil given.

The more nearly the mean increases in each curve lie in a straight line when plotted against the logarithm of the dose, the more truly is the response expressed by the corresponding logarithmic equation.

line through the five points was obtained by the method described by Fisher [1925, p. 117].

$$y = a + b(x - \bar{x})$$

where

$$a = \bar{y} = \text{mean value of } y$$

$$\bar{x} = \text{mean value of } x$$

and

$$b = \frac{\sum y(x - \bar{x})}{\sum (x - \bar{x})^2}.$$

This gave the equation $y = 12.1 + 40.24x$ (where x is the logarithm of the dose of oil), whence is derived the equation $y = 12.1 + 40.24 \log x$ to represent the relation between response and dose of oil.

This equation was obtained from results on groups of rats consisting of approximately equal numbers of bucks and does. It occurred to us that it might be useful to have separate equations for results from bucks and does respectively. We therefore plotted the mean increases of the bucks and does in each group separately against the logs of the doses. These also gave two practically straight lines (Fig. 1) which were determined by the same method:

$$y = 11.3 + 50.3x \text{ (bucks),}$$

and

$$y = 12.4 + 27.4x \text{ (does).}$$

The equations relating response to dose are therefore:

$$y = 11.3 + 50.3 \log x \text{ (bucks),}$$

and

$$y = 12.4 + 27.4 \log x \text{ (does).}$$

Two points of interest arise from a consideration of these curves. Firstly, only about 15 animals were used for each point on the curve. The smoothness of the curve would appear to indicate therefore that a very reliable average can be obtained from 15 animals, a comparatively small number for a biological estimation. Secondly, the two curves indicate that the bucks respond more vigorously than the does to the higher doses of vitamin A but it is remarkable that the does respond more vigorously than the bucks to the lower doses. This led us to wonder whether the mortality among the bucks had been greater than that among the does in work on vitamin A deficiency. We therefore counted the numbers of bucks and does respectively that had died during the test period (5 weeks) of the experiment and also those from similar experiments that we have just concluded. We found that, of 288 bucks which had been used for testing various doses of cod-liver oil, 57 or 19.8 % had died, while of 277 does used in the same experiments (in more or less equal numbers on each test) 37 or 13.4 % had died. This difference appears to be significant.

Dr J. Gaddum of the National Institute for Medical Research has made the following calculation from the data and has concluded that the difference is significant and, therefore, that male rats require more vitamin A to prevent death than do female rats.

Group of rats	Dose of cod-liver oil given daily mg.	No. of bucks in group	Total no. of bucks dying during test	% no. of bucks dying during test	No. of does in group	Total no. of does dying during test	% no. of does dying during test
1	1 oil B	12	2	16.7	10	1	10.0
2	4 " B	5	1	20.0	5	0	0.0
3	0.5 " C	11	4	36.4	14	2	14.3
4	1.0 " C	9	3	33.3	14	1	7.1
5	5.0 " C	14	1	7.1	4	0	0.0
6	5.0 " C	7	0	0.0	12	1	8.3
7	0.5 " D	9	1	11.1	13	1	7.7
8	1.0 " D	10	2	20.0	13	2	15.4
9	0.5 " E	13	3	23.1	16	0	0.0
10	1.0 " E	13	3	23.1	13	2	15.4
11	0.1 " F	17	7	41.2	11	0	0.0
12	0.5 " F	9	1	11.1	13	1	7.7
13	1.0 " F	16	2	12.5	6	0	0.0
14	1.0 " G	11	3	27.3	11	3	27.3
15	1.0 " H	10	0	0.0	12	3	25.0
16	1.0 " J	11	1	9.1	12	0	0.0
17	1.0 " K	2	0	0.0	9	1	11.1
18	0.25 " A	18	11	61.1	13	6	46.2
19	1.0 " A	23	6	26.1	14	7	50.0
20	1.5 " A	13	3	23.1	22	5	22.7
21	2.5 " A	21	2	9.5	11	0	0.0
22	7.5 " A	19	1	5.3	12	0	0.0
23	20.0 " A	15	0	0.0	17	1	5.9
288			57		277	37	

If the sexes were equally sensitive, the number of groups in which the mortality of the males was larger than that of the females should equal the number of groups in which the mortality of the females was larger than that of the males. Actually, more males than females died in 17 groups, more females than males died in 5 other groups and in 1 group the numbers of males and females that died were equal. Thus:

Observed	...	...	...	...	...	...	More males dead	More females dead
Calculated on assumption that mortalities are equal							17.5 (a)	5.5 (b)
							A (c)	A (d)

where A is a very large number.

To find the probability that these two results are compatible [see Fisher, 1925, p. 84]:

$$\begin{aligned}
 \chi^2 &= \frac{(ad - bc)^2 (a + b + c + d)}{(a + b)(c + d)(a + c)(b + d)} \\
 &= \frac{[A(17.5 - 5.5)]^2 (2A)}{23 \times 2A \times A \times A} \quad (\text{neglecting } a + b \text{ in comparison with the large number } A) \\
 &= \frac{12^2}{23} = 6.26.
 \end{aligned}$$

The table of χ^2 shows (when $n = 1$) that the probability of this value of χ^2 being due to chance is between 1/50 and 1/100.