CLV. THE INFLUENCE OF DIFFERENT CASEIN PREPARATIONS IN RIBOFLAVIN-DEFICIENT DIETS UPON THE APPEARANCE OF CATARACT¹

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(Received 30 May 1938)

OUR experiments showing that nutritional cataract in rats can be prevented by riboflavin [Day *et al.* 1937] and in its early stages can be arrested by riboflavin [Day *et al.* 1938] would seem to solve the problem of the aetiology of this experimental condition. Although there is now no doubt that cataract can readily be produced by riboflavin deficiency under suitable conditions, there appear still to be unanswered questions regarding the exact conditions that must be met to produce this experimental state. By following our experimental procedures O'Brien [1932] and Yudkin [1933] were able to produce cataract in a high percentage of cases. On the other hand, Bourne & Pyke [1935] observed a maximum incidence of only 31 % cataract in their groups of rats. Without giving the details of all his experiments, György [1935] stated: "In an extensive series of experiments in which over 500 rats were involved, we never observed this phenomenon (cataract), either with complete or partial deficiency of the vitamin B₂ complex or with separate deficiencies of lactoflavin or vitamin B₆."

Several years ago we observed that altering the type of casein preparation in our experimental diet caused a noticeable change in the time of appearance of cataract. We therefore obtained samples of casein from different sources, and used them in riboflavin-deficient diets. Our results are interesting in relation to the problem of cataract production by riboflavin deficiency. They may also prove of interest to workers in other fields of nutrition, since casein is a common constituent of experimental diets.

EXPERIMENTAL

All the animals used in these experiments were reared under standardized conditions in our own laboratory. The stock diet used was Sherman's diet 13 [Sherman & Campbell, 1924], which is composed of one-third whole milk powder, two-thirds ground whole wheat and NaCl. This diet was fed *ad lib.* and was supplemented twice a week with fresh lettuce and beef heart. The young were placed on the experimental diet at 21 days of age, and only litters containing 8 or more young were used. Weekly ophthalmoscopic examinations were made and the animals were kept until death.

The experimental riboflavin-deficient diet (No. 625) had the following composition: casein, 18 %; cod liver oil, 2 %; butter fat, 8 %; salt mixture [Osborne & Mendel, 1919], 4 %; and corn (maize) starch, 68 %. The corn starch carried an

¹ Research paper No. 512, journal series, University of Arkansas. Read before the meeting of the American Society of Biological Chemists, 21-24 April 1937.

extract of rice polish prepared by the procedure described by Day & Langston [1934]. All the animals in this series received diets of this composition, differing only in the source and treatment of the casein. Several casein preparations were used which are described in the following paragraphs.

I. *Alcohol-extracted*. "Casein, edible, muriatic" (Casein Manufacturing Co. of America), a crude HCl-precipitated casein, which was washed with 60 % alcohol in this laboratory, according to the technique of Sherman & Spohn [1923].

II. *Dilute acid-extracted*. "Casein, edible, muriatic", subjected to prolonged washing with acidulated water, according to the method described by McCollum *et al.* [1922].

III. *Labco*. A "vitamin-free" casein (Casein Manufacturing Co.). The method of manufacture has been described briefly by Supplee *et al.* [1936]. It has been used in a number of experiments in this laboratory [Day & Darby, 1936; Day *et al.* 1937; Day *et al.* 1938].

IV. *Harris*. Described by the manufacturers (Harris Laboratories, New York) as a "vitamin-free" casein. The method of preparation is unknown to us.

V. *Glaxo AB*. Glaxo caseinogen AB (Glaxo Laboratories, Ltd., batch F46), described by the manufacturer as "a precipitated acid-washed heat-treated ashless casein". It has been widely used, presumably without further treatment, in many experiments upon the components of the vitamin B complex [Birch *et al.* 1935; György, 1935; Harris, 1935; Prunty & Roscoe, 1935; Ray *et al.* 1935].

VI. *Glaxo ABE*. Glaxo caseinogen ABE (batch F45/E) which the manufacturers state has replaced the Glaxo AB preparation for vitamin A and B experiments. It was described as "heat-treated 'ashless' casein, alcohol-extracted after heat treatment to the maximum possible removal of ether-soluble matter".

VII. *Glaxo ABE—alcohol-extracted*. Glaxo caseinogen ABE subjected to treatment with 60 % alcohol in this laboratory, following the method of Sherman & Spohn [1923].

RESULTS AND DISCUSSION

Table I gives a summary of our data and Fig. 1 represents a part of the same data in graphic form. The acid-extracted and Harris caseins gave rates of growth, periods of survival, and times for the appearance of cataract almost identical with those given by the Labco casein, as may be seen from a study of the table. For this reason, and also since superimposed lines on the chart would have been confusing, the curves for these two groups were omitted from the chart.

There are 5 obvious criteria that may be used in a study of these data for comparing the 7 casein preparations: (1) average maximum gain, (2) average time to reach maximum weight, (3) average time of appearance of cataract, (4) incidence of cataract and (5) average survival period. Judged by all 5 criteria, the acid-extracted, Labco, and Harris caseins gave almost identical results. It is simplest to assume that these 3 caseins were essentially riboflavin-free, although an alternate explanation is that they all contained the same small quantity of riboflavin.

On the basis of 4 out of the 5 criteria the alcohol-extracted casein appeared to contain a small but significant amount of riboflavin. Although the numbers of animals are not great enough for the data to be treated statistically, we believe that there is significance in the longer survival, delayed appearance of cataract and more prolonged growth in animals receiving this casein; many of the animals receiving the alcohol-extracted and Labco casein diets were litter-mates, carefully matched as to size and sex.

The two Glaxo casein diets gave much greater growth, the growth was more prolonged, the average survival period was significantly greater and the

Table I. *Summary of data regarding growth, survival and incidence and time of appearance of cataract in groups of rats receiving riboflavin-deficient diets containing different casein preparations*

	No. of rats	No. showing cataract	Av. wt. at start g.	Av. max. gain g.	Av. no. days to reach max. wt.	Av. no. days for appearance of cataract	Av. no. days survival
I. Alcohol-extracted	15	13	41.1	17	37	83	119
II. Acid-extracted	9	8	39.9	20	28	56	81
III. Labco	17	12	39.6	16	33	50	90
IV. Harris	9	8	37.6	16	23	52	83
V. Glaxo AB	10	7	38.5	42	65	113	133
VI. Glaxo ABE	14	6	38.1	40	62	86	117
VII. Glaxo ABE extracted	5	2	35.8	22	43	75	101

Fig. 1. Composite weight curves of groups of rats which received riboflavin-deficient diets identical in all respects except for the caseins that they contained. The numbers on the curves indicate the number of rats in each group. The asterisks mark the time of appearance of cataract in individual animals, and the daggers mark the death of individual animals. Each curve was terminated at the point of average survival for that group; consequently, animals showing cataract and those dying after that time are not indicated on the chart. The dotted line indicates the average growth of a group of rats receiving the Labco casein diet supplemented with 30% riboflavin weekly; these animals were killed at the end of 15 weeks.

appearance of cataract was delayed. Furthermore, the percentage incidence of cataract was lower among rats receiving these casein preparations. Whereas the alcohol-extracted casein gave an incidence of 87%, the Labco 71%, the Harris 89% and the acid-extracted 89% of cataracts, the group of rats receiving the Glaxo AB casein showed an incidence of 70% and the Glaxo ABE animals only 43% of cataracts. The incidence for the combined groups receiving Glaxo AB and ABE was 54%. A consideration of the incidences of cataract for the first 10 weeks of experiment only is even more revealing. They were as follows: alcohol-extracted, 47%; acid-extracted, 89%; Labco, 59%; Harris, 89%; Glaxo AB, 10%; and Glaxo ABE, 14%. Thus, judged by all 5 criteria, the two Glaxo caseins appeared to contain significant amounts of riboflavin.

The seventh sample of casein used—designated in the table as Glaxo ABE extracted—was some of the Glaxo ABE casein subjected to extraction with 60% alcohol in this laboratory, by the method employed in preparing the alcohol-extracted casein. Unfortunately, there was sufficient material to feed only 5 rats and this experiment was run after most of the animals in the other groups had

died, so this series was not well controlled. However, it is evident that growth and survival were approximately the same as in the group receiving the alcohol-extracted casein.

This evidence of a biological nature indicating the presence of riboflavin in two of the casein samples was confirmed by physical means. Small samples of the caseins were shaken with distilled water and the clear eluates examined under ultraviolet light in a dark room. The source of light was an "Eveready Fluoray" lamp, which emits an intense ultraviolet radiation practically free from visible light. Dilute solutions of riboflavin give a bright greenish fluorescence when placed in this "black light" [Supplee *et al.* 1936]. Eluates from the alcohol-extracted, acid-extracted and Harris caseins gave no fluorescence. An eluate from the Labco casein gave a bluish fluorescence, quite different in colour from that due to riboflavin. However, eluates from the two Glaxo caseins emitted a marked greenish fluorescence, similar to the fluorescence of a pure riboflavin solution, indicating the probable presence of this substance.

Complete riboflavin deficiency results in a pathological picture in the young rat rather different from that seen in partial riboflavin deficiency. The rats receiving acid-extracted, Labco and Harris caseins developed an unkempt appearance of the hair and some alopecia, but extensive alopecia was uncommon and marked involvement of the skin was rare. As shown above, cataract (and also keratitis) was seen in nearly all the animals. This is a typical picture of what we believe to be complete riboflavin deficiency in young rats. On the other hand, animals receiving a small amount of riboflavin—e.g. 30 γ a week—show an extensive loss of hair; the body and head frequently become quite denuded. In many animals dry eschars form on various parts of the body, particularly on the face and head. Excoriation of the face and severe ophthalmia are common. Severe keratitis is found in all such animals, but cataract is less common. This latter is the picture that was seen in the animals receiving the Glaxo caseins.

The data presented in this report would seem to indicate that the failure of some investigators to observe cataract in riboflavin deficiency may have been due to the contamination of their caseins with riboflavin. This is probably not the whole explanation of such failure, however. It is probable that the size of animals, previous dietary history, and other conditions also influence the susceptibility of rats to cataract in riboflavin deficiency. Some of these factors are now being studied.

SUMMARY

Seven casein preparations were used as sources of protein in riboflavin-deficient diets. The responses of groups of rats fed these diets were compared by 5 criteria: average maximum growth, average time required to reach maximum weight, average time of appearance of cataract, incidence of cataract and average period of survival. Two commercial "vitamin-free" caseins and a crude casein washed with acidulated water gave nearly identical responses as judged by these 5 criteria. A crude casein washed with dilute alcohol gave slightly longer growth and survival, and the appearance of cataract was somewhat delayed. It is believed that washing with dilute alcohol failed to remove the last trace of riboflavin. Two commercial purified caseins that have been widely used in experiments upon the B-vitamins promoted much greater and longer continued growth, and permitted longer survival. The groups of rats receiving these two casein diets exhibited lower incidences of cataract, and the appearance of cataract was definitely delayed. Thus, judged by all 5 criteria, these two casein preparations appeared to contain significant amounts of riboflavin. Furthermore, eluates

from these two caseins exhibited a marked greenish fluorescence, similar to that produced by riboflavin, when examined in a dark room under ultraviolet light. It is believed that the failure of some investigators to observe cataract in riboflavin deficiency may have been due, in part, to the contamination of their caseins with riboflavin.

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