# QUANTITATIVE RELATIONSHIPS BETWEEN ADMINISTERED CHOLESTEROL AND ALFALFA REQUIRED TO PREVENT HYPERCHOLESTEROLAEMIA IN RABBITS

F. B. COOKSON* AND S. FEDOROFF

*From the Departments of Anatomy, University of Alberta, Edmonton, Alberta (Canada) and University of Saskatchewan, Saskatoon, Saskatchewan (Canada)*

Received for publication February 12, 1968

In previous experiments (Cookson, Altschul and Fedoroff, 1967), we have shown that the hypercholesterolaemia which follows the oral administration of cholesterol to rabbits can be prevented by simultaneously feeding the animals a diet consisting mainly of alfalfa. Furthermore, Horlick, Cookson and Fedoroff (1967) have shown that alfalfa exerts this effect by interfering with the absorption of cholesterol by the gut. In these early experiments the diet used consisted of 90 per cent alfalfa and 10 per cent calf meal and the cholesterol administered was a standard daily dose of 0·6 g. The experiments reported here were undertaken to determine whether or not there is a relationship between the amount of cholesterol administered and the amount of alfalfa necessary to prevent a rise in the serum cholesterol.

TABLE I.—*Composition of Diets*

| (1) *Standard Calf Meal Diet*† | Weight lb. |
|---|---|
| Oats No. 1 | 350·0 |
| Wheat | 460·0 |
| Soya Bean Meal | 100·0 |
| Calcium Phosphate | 9·0 |
| Vitamin A premixed | 1·0 |
| Vitamin D premixed | 0·1 |
| Vitamin E premixed | 2·5 |
| Iodized salt | 5·0 |
| Beet molasses | 50·0 |
| Brewers yeast | 20·0 |
| TOTAL | 997·6 |
| (2) *25 per cent Experimental Alfalfa Diet*† | Weight lb. |
| Alfalfa | 25 |
| Standard calf meal diet | 75 |
| TOTAL | 100 |
| (3) *50 per cent Experimental Alfalfa Diet*† | Weight lb. |
| Alfalfa | 50 |
| Standard calf meal diet | 50 |
| TOTAL | 100 |
| (4) *75 per cent Experimental Alfalfa Diet*† | Weight lb. |
| Alfalfa | 75 |
| Standard calf meal diet | 25 |
| TOTAL | 100 |

† Mixtures were made up into pellet form

* Present address: Department of Anatomy, University of Alberta, Edmonton, Alberta (Canada).

## MATERIALS AND METHODS

Fully mature rabbits weighing over 3·0 kg. were used as experimental animals. The cholesterol was given by mouth in gelatin capsules on 6 days a week.

In order to ensure that the intake of alfalfa varied in different animals without restricting the actual food intake, experimental diets were made up containing different percentages of alfalfa. Four diets were used and their compositions are given in Table I. A range of doses of cholesterol was used and the animals were divided into 4 groups with respect to this. Each group was then divided into 4 subgroups with respect to diet. Table II shows this management

TABLE II.—*Management of Animals with Respect to Cholesterol Dose Given and Dietary Regime*

| Group No. | Daily dose of Cholesterol (g.) | Sub-group No. | Dietary regime |
|---|---|---|---|
| A | 0·3 | 1 | Standard calf meal diet |
| | | 2 | 25 per cent Alfalfa diet |
| | | 3 | 50 per cent Alfalfa diet |
| | | 4 | 75 per cent Alfalfa diet |
| B | 0·4 | 1 | Standard calf meal diet |
| | | 2 | 25 per cent Alfalfa diet |
| | | 3 | 50 per cent Alfalfa diet |
| | | 4 | 75 per cent Alfalfa diet |
| C | 0·5 | 1 | Standard calf meal diet |
| | | 2 | 25 per cent Alfalfa diet |
| | | 3 | 50 per cent Alfalfa diet |
| | | 4 | 75 per cent Alfalfa diet |
| D | 0·6 | 1 | Standard calf meal diet |
| | | 2 | 25 per cent Alfalfa diet |
| | | 3 | 50 per cent Alfalfa diet |
| | | 4 | 75 per cent Alfalfa diet |

and the regime to which the animals in the various groups and subgroups were subjected. In the case of the control animals, receiving the standard calf meal diet only, the experiments were conducted for periods of 8 weeks, with the others the experiments were conducted for 16 weeks. The diets were given *ad libitum* and the intake of each animal recorded daily. The weight of each animal was recorded weekly.

The sex of each animal, although noted, was not taken into account in assessing results, for previous experiments and other workers (Altschul, 1950) have indicated that individual variations in response are of greater significance than variations which can be attributed to sex.

The method used to determine the serum cholesterols was that described by Bowman and Wolf (1962). Estimations were carried out at weekly intervals on each animal for 3 weeks prior to the start of the experiments and then throughout the experimental period.

## RESULTS

The experiments were continued until complete data had been collected from 5 animals in each subgroup *i.e.* from 20 animals in each group. Data from animals not completing their experimental period as a result of intercurrent infection or similar factors were not used. Substitutes for such animals were incorporated in the the experimental groups. In all 11 animals were replaced. Fig. 1 shows graphically the mean values of the results from the control animals in each group, sub-groups I. These animals were fed a diet containing no alfalfa and the expected pattern of rise in serum cholesterol levels is seen. From a lag phase of up to 2 weeks from the start of the experimental period the serum cholesterol levels rise to a high value and then plateau. The maximum level reached differs but in general is higher the greater the dose of cholesterol given.

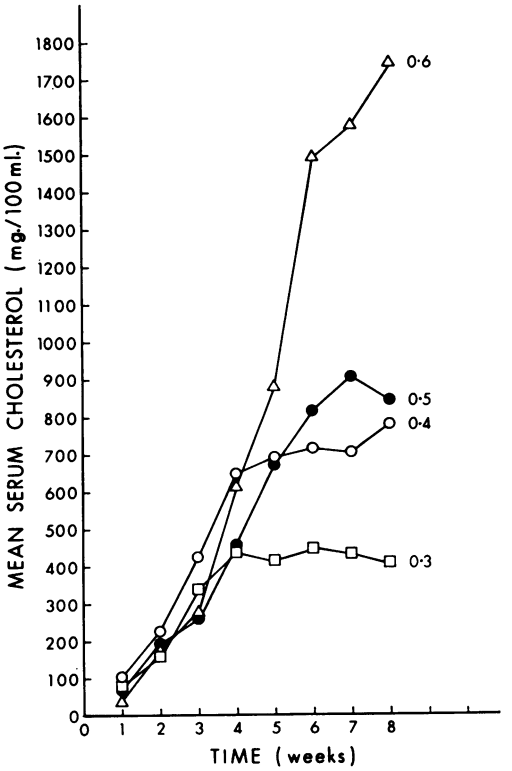


FIG. 1.—Results from the control animals, all groups, subgroup 1. The mean serum cholesterol levels of the animals at each dose level of cholesterol are shown at weekly intervals. Figures on the right represent the daily cholesterol intake in grams.

In the animals receiving the alfalfa diets, elevation of the serum cholesterol levels above the upper limit of normal for rabbits (150 mg./100 ml.) occurred only in those animals receiving the 25 per cent alfalfa diet (subgroups 2). All animals in these subgroups were affected at some time during the experimental period.

Table III shows an analysis of the result from the animals in the 4 groups receiving the 50 per cent and 75 per cent alfalfa diets (subgroups 3 and 4). In no

TABLE III.—*Serum Cholesterol Levels in Animals Receiving 50 and 75 per cent Alfalfa Diets*

| Group No. | Sub-group No. | Mean of mean serum cholesterol values (mg./100 ml.) | Range of mean serum cholesterol values (mg/100 ml.) | Mean of mean alfalfa intakes (g./week) | Range of mean alfalfa intakes (g. week) |
|---|---|---|---|---|---|
| A | 3 | 71 | 51– 83 | 433 | 350–560 |
| | 4 | 69·4 | 30– 96 | 699 | 620–831 |
| B | 3 | 64·4 | 46– 82 | 415 | 374–427 |
| | 4 | 73 | 57– 98 | 785 | 662–966 |
| C | 3 | 84 | 69– 94 | 419 | 339–510 |
| | 4 | 75·4 | 67–103 | 856 | 675–1013 |
| D | 3 | 85 | 69–105 | 461 | 398–568 |
| | 4 | 62·8 | 45– 72 | 692 | 596–802 |

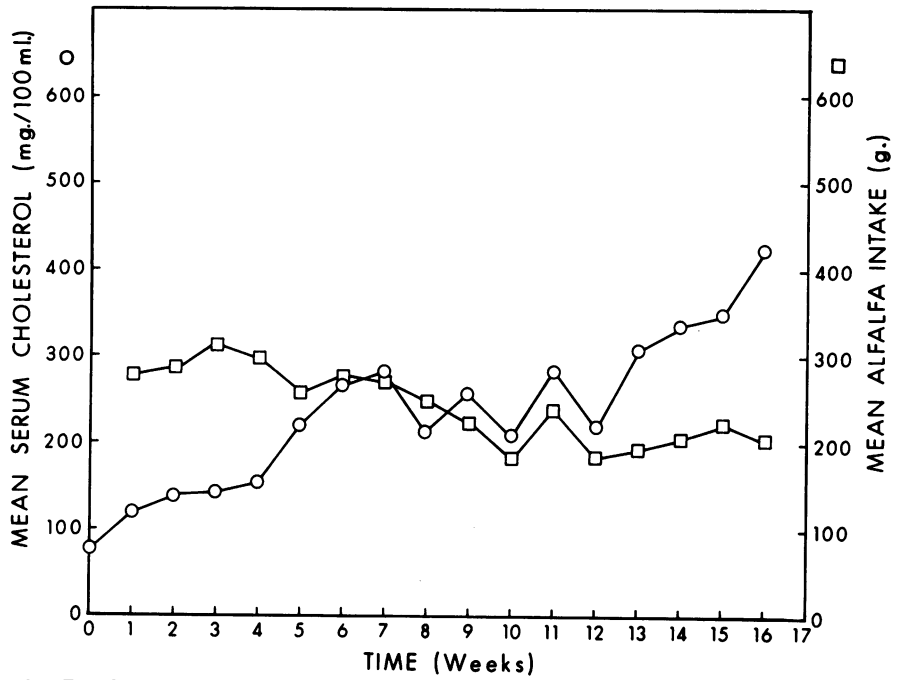


FIG. 2.—Results from the animals of Group A, subgroup 2. Mean serum cholesterol levels and mean alfalfa intakes from all animals are shown at weekly intervals.

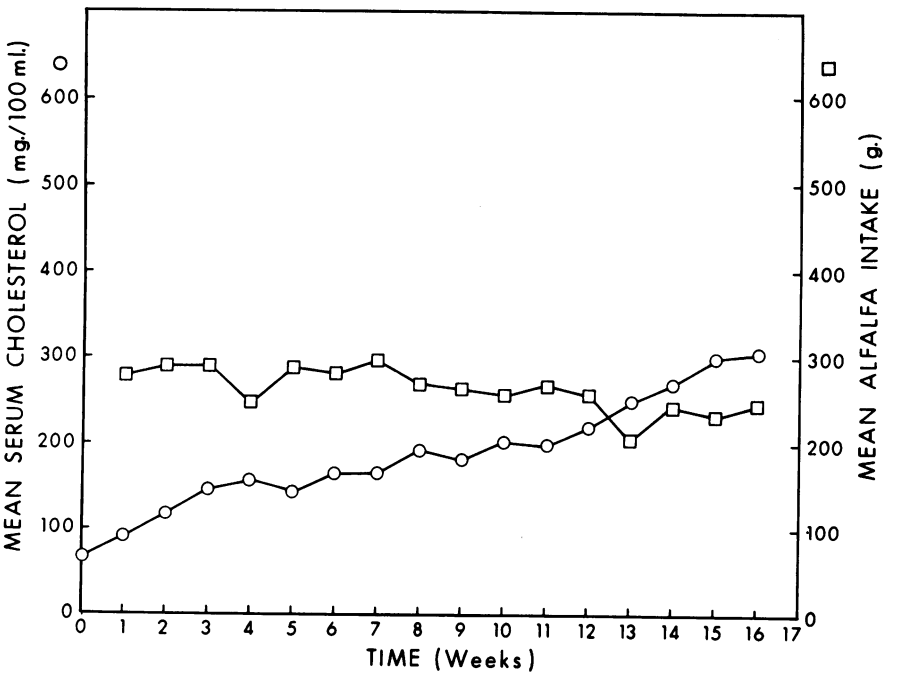


FIG. 3.—Results from the animals of Group B, subgroup 2. Mean serum cholesterol levels and mean alfalfa intakes from all animals are shown at weakly intervals.

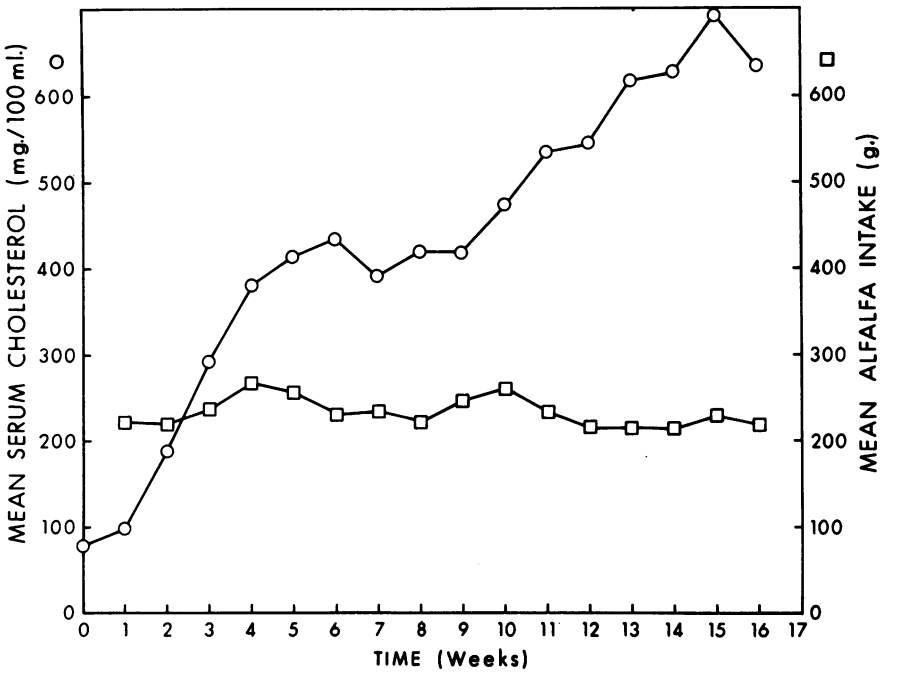


FIG. 4.—Results from the animals of Group C, subgroup 2. Mean serum cholesterol levels and mean alfalfa intakes for all animals are shown at weekly intervals.

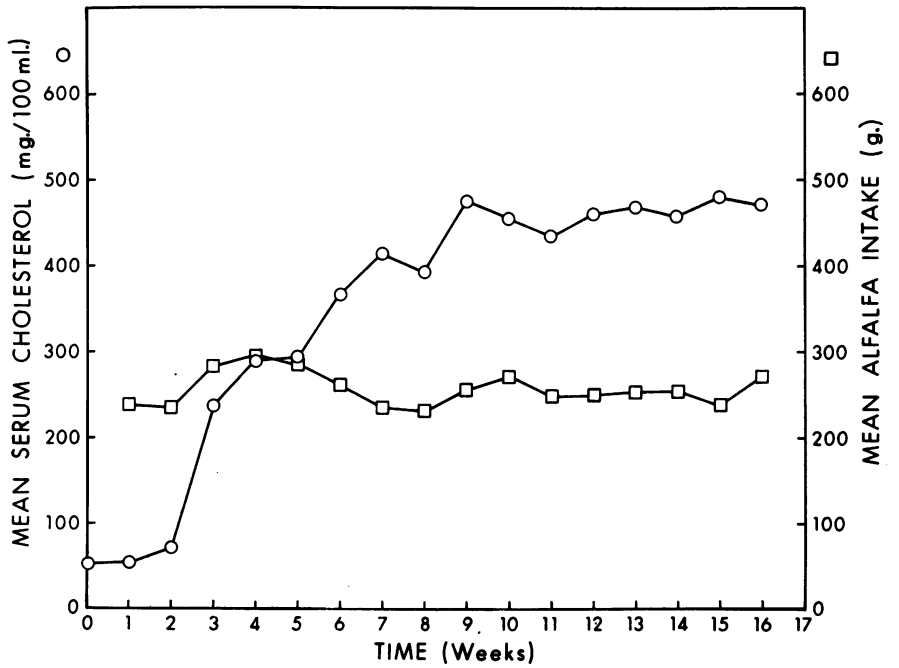


FIG. 5.—Results from the animals of Group D, subgroup 2. Mean serum cholesterol levels and mean alfalfa intakes for all animals are shown at weekly intervals.

animal did elevation of the serum cholesterol level above the upper limit of normal occur, indeed in many the mean value of this level was depressed below the resting values for the animal concerned and below the limits of the range of serum cholesterol values normally observed in our animals in the resting state (70–150 mg./100 ml.).

Figs 2, 3, 4 and 5 show graphic analyses of the results from the animals in which serum cholesterol elevation did occur during the experimental period. Fig. 2 is obtained from animals in group A, Fig. 3 group B, Fig. 4 group C and Fig. 5 group D. In these figures the mean serum cholesterol from the 5 animals involved is plotted at weekly intervals together with the mean alfalfa intake. Two points are apparent from study of these graphs.

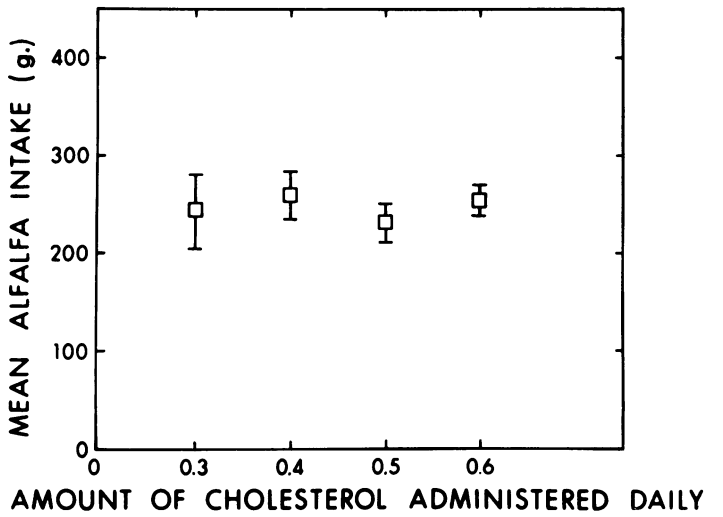


FIG. 6.—Mean alfalfa intakes over the total experimental period against daily cholesterol dosage for all the animals in subgroup 2 of each group. Standard deviations are shown.

1. Elevation of serum cholesterol above the upper limit of normal occurs in these animals when the intake of alfalfa falls below about 300 g. per week.

2. The level of alfalfa intake below which serum cholesterol elevation occurs appears to be independant of the dose of cholesterol given.

Fig. 6 further demonstrates the second point. The mean alfalfa intake of the animals over the total experimental period is plotted at each dose level of cholesterol. Standard deviations are shown and it is seen that there is no significant difference between these mean values and deviations.

## DISCUSSION

The results given above together with our previous finding, that alfalfa prevents the absorption of cholesterol by the gut (Horlick *et al.*, 1967), allow certain deductions to be made as to exactly how alfalfa prevents hypercholesterolaemia. Three mechanisms of action may be suggested:

1. The effect may be a bulk effect of undigested fibres.

2. Some component of alfalfa may be forming inabsorbable complexes with cholesterol in the gut.

3. Some component of alfalfa may be blocking the absorptive mechanism for cholesterol in the intestinal mucosa.

The first possibility can be eliminated by the results presented in conjunction with certain other evidence. Rabbit diets which do not contain alfalfa, such as our calf meal diet, have no apparent protective effect against administered cholesterol. A wide variety of diets, the majority containing some alfalfa, have been used by other workers in this field (Constantinides, 1965) but effects attributable to differences in residue of undigested fibre have not been reported. It would be expected that any effect of undigested fibre to prevent cholesterol absorption would be strongly dose related to the cholesterol administered. The very opposite of such a relationship is seen in our results. Finally, with our animals, the amount of faeces excreted daily by those on the alfalfa diets was not greater than that excreted by those on the control diet. A decision between which of the 2 remaining mechanisms is involved in preventing cholesterol absorption or whether both are acting together is somewhat more difficult and no definite answer can be given from the results of the experiments reported here. The observation that the same amounts of alfalfa are necessary to protect the animals from any of the doses of cholesterol given seems to favour the third mechanism cited, that is that some component of alfalfa may be blocking the absorptive mechanism for cholesterol in the intestinal mucosa. In a situation in which inabsorbable complexes were being formed a progressive relationship between the dose of cholesterol given and the amount of alfalfa necessary for protection against hypercholesterolaemia would be expected.

It could be suggested that the effect of alfalfa in preventing hypercholesterolaemia is merely an expression of the $\beta$-sitosterol content of the plant. Even if this were so it would still not clarify which of mechanisms 2 and 3 cited above was operative. Whilst some workers (Glover, Leat and Morton, 1957; Glover and Morton, 1958) consider that competition for mucoprotein and lipoprotein attachment sites is the mechanism by which $\beta$-sitosterol prevents the absorption of cholesterol others (Pollack, 1953; Hudson, Diller, Pfeiffer and Davies, 1959) suggest that this phytosterol forms inabsorbable complexes with cholesterol in the gut. However, it has been shown (Pollack, 1953) that approximately 7 times as much $\beta$-sitosterol as cholesterol is necessary for the phytosterol to prevent completely the absorption of the cholesterol. We have measured the total amounts of phytosterols present in our samples of alfalfa and found not more than 70 mg./100 g. of alfalfa. Patently this is far too little to ascribe the observed effects of alfalfa to its $\beta$-sitosterol content.

Finally, whilst our results demonstrate the protective effects of alfalfa for daily doses of cholesterol up to 0·6 g. it cannot be assumed that this protection extends to higher cholesterol doses. Parker and Odland (1966) used a pure alfalfa diet in their experiments but this was impregnated with 3–4 g. of cholesterol per 100 g. of food. They obtained extremely high levels of serum cholesterol in their animals (1200 mg./100 ml.). Presumably there is an upper limit of cholesterol dose in rabbits against which the active component of alfalfa can protect. Again this appears to support the view expressed above that the active component of alfalfa exerts its effects by block of the cholesterol absorptive mechanisms of the gut.

SUMMARY

The results of experiments designed to investigate quantitative relationships between cholesterol administered and the amount of alfalfa necessary to prevent hypercholesterolaemia are described.

Alfalfa was found to be very efficient in this respect. The ingestion of approximately 300 g. per week was sufficient to protect a rabbit completely from the adverse effects of daily doses of cholesterol up to and including 0·6 g.

Analysis of all the experimental results indicate that alfalfa acts either: (1) by some component forming inabsorbable complexes with cholesterol in the gut, or (2) by some component blocking the absorptive mechanism for cholesterol in the intestinal mucosa. The weight of the evidence appears to favour the latter alternative.

The possibility that the observed effects of alfalfa feeding may be an expression of the $\beta$-sitosterol content of the plant are discounted.

This work was supported by the Canadian Medical Research Council Grant M.A.–1957 and by the Saskatchewan and Alberta Heart Foundations.

## REFERENCES

ALTSCHUL, R.—(1950) Selected studies in Arteriosclerosis. Springfield, (Charles C. Thomas), p. 7.

BOWMAN, R. E. AND WOLF, R. C.—(1962) *Clin. Chem.*, **8**, 302.

CONSTANTINIDES, P.—(1965) ' Experimental Atherosclerosis. ' Amsterdam (Elsevier), p. 44.

COOKSON, F. B., ALTSCHUL, R., AND FEDOROFF, S.—(1967) *J. Atheroscler. Res.*, **7**, 69.

GLOVER, J., LEAT, W. M. F., AND MORTON, R. A.—(1957) *Biochem. J.*, **66**, 214.

GLOVER, J., AND MORTON, R. A.—(1958) *Br. med. Bull.*, **14**, 226.

HORLICK, L., COOKSON, F. B., AND FEDOROFF, S.—(1967) *Circulation*, **36**, II 18.

HUDSON, J. L., DILLER, E. R., PFEIFFER, R. R., AND DAVIES, W. W.—(1959) *Proc. Soc. exp. Biol. Med.*, **102**, 461.

PARKER, F., AND ODLAND, G. F.—(1966) *Am. J. Path.*, **48**, 197.

POLLACK, O. J.—(1953) *Circulation*, **7**, 696.