Lipotropic Factors in Transport of Cholesterol in Experimental Animals

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At Dr. Hartroft's suggestion I am reporting some of the experiments that Dr. Jessie Ridout and Dr. Jean Patterson and I did a few years ago as well as some more recent work.

We were not primarily interested in the blood picture but were working on fatty livers. Becoming aware of the suggestion that there was possibly a causal relationship between blood cholesterol levels and the incidence of atherosclerosis, we wondered just what choline, betaine or inositol might do in this situation.

Some of the rats from our usual liver studies were killed by exsanguination and the blood was analysed for cholesterol using the Schonheimer-Sperry-Webb[1] procedure. Thus, we obtained a single value at the end. Sometimes we pooled the samples. With no standard deviations, we did not know whether the differences were real or not. However, as time went on, certain facts and impressions emerged.

The time after the last meal at which one collects the blood sample is important, especially when the diet contains cholesterol and lipotropic agents. In our colony, when the blood of young rats (70 to 150 gm., maintained on a commercial ration) was taken eighteen hours or more after their last meal, total cholesterol in the serum was about 70 (±S.D. 11) mg. per 100 ml. with about 12 mg. in the free form; the rest was bound in ester form. Young males and females showed no significant difference.

When purified diets containing cholesterol and choline were fed to rats a distinct postprandial elevation in bound cholesterol was observed. The values were well above normal about two to four hours after eating and fell back in about sixteen to eighteen hours into the normal range and stayed there. No matter how much cholesterol was in the diet, even up to 1.6 per cent, if there was no choline in the diet, no significant elevation of the serum cholesterol occurred. When the food contained both choline and cholesterol there was a rather sharp rise which is related to the amount of cholesterol but not to the concentration of choline in the diet, as long as some was present.[2]

In the absence of choline no increase occurred in the serum cholesterol. This suggested, of course, that choline affects absorption of cholesterol but other explanations are possible and the data that we had available would not permit us to resolve this.

When the rats were kept on a hypolipotropic diet for any length of time the bound serum cholesterol fell progressively as the experiment was prolonged. In one experiment, after about six months the values had fallen to about 30 mg. per 100 ml. or even lower. However, addition of quite small amounts (0.02 per cent) of choline or betaine to the food maintained the serum cholesterol within the normal range. The administration of inositol, even at fairly high levels, failed completely to prevent this characteristic fall in the concentration of cholesterol in serum.

When choline was restored to the diet of rats with fatty livers and low serum cholesterol, the latter returned promptly from about 30 mg. per 100 ml. to 65 to 75, i.e., to the normal level. This happened within twenty-four hours, long before we could detect any chemical or histological change in the liver. Betaine had a similar

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effect. The addition of inositol failed to bring about any rise.

Vitamin B_{12} was not tested under these conditions but both betaine and vitamin B_{12}, like choline, prevent the fall even when the animals are maintained on this ration for long periods. There were far too many uncontrolled variables in those early experiments. We tried to eliminate them one after another.

We had just started to investigate the effect of repeated withdrawal of blood upon serum cholesterol when I discovered that in several other laboratories, a gradual progressive change upward had been noted. We decided not to use the same animals for repeated testing because the results so obtained might be misleading unless we could find some better micro method. Fortunately, a more suitable method is now available, thanks to Zlatkis, Zak and their colleagues.[3,4] Using a reagent containing ferric chloride in concentrated sulfuric acid, reasonably accurate values can be obtained for total cholesterol on as little as 0.1 ml. of serum. We collect about 0.5 ml. of blood by snipping a tiny portion off the tail of a rat. If bleedings are limited to five or six in a period of forty-eight hours and the rat is not bled for a period of a couple of weeks, we find no progressive rise in the serum cholesterol.

When this method was used on the blood from large male rats (weighing between 400 to 500 gm.) the mean value for eighty-one samples from fifteen animals was 83 (± S.D. 19) mg. per 100 ml.

When these large rats, which had been on a standard chow diet, were given an injection of 16 mg. choline chloride, no change whatever occurred in the serum cholesterol at one, two, four, eight, twenty-four or forty-eight hours.

In one experiment with young rats (weighing 150 gm.) we studied the effect of ten different dietary fats fed for three weeks at the 20 per cent level in diets with six different amounts of choline upon the serum cholesterol.[5] The fats used were sunflower seed oil, corn oil, peanut oil, olive oil, Primex,® margarine, lard, beef fat, butter fat and coconut oil. In every case the serum cholesterol levels fell below normal when choline was omitted from the ration.

As little as 0.03 per cent choline in the food maintained the serum cholesterol values at about normal, with one or two exceptions. Rats getting butter and coconut oil required slightly over 0.03 per cent.

Higher doses of choline (up to 0.36 per cent) brought the serum cholesterol level back into the normal range and left it there. The different fats did not produce different levels of serum cholesterol, whether choline was present or not.

The big rats (those weighing 400 gm.) were maintained for a relatively long period (twenty-four weeks) on a hypolipotropic diet. The serum cholesterol level was then determined on the animals after they had been without food for eighteen hours. The values were low (30 to 60 mg. per 100 ml.), as we expected. The serums of the same rats were analyzed again after a few days, and a few weeks later, with remarkably good agreement, i.e., the values were on a plateau.

When these rats ate 1 to 5 gm. of choline-deficient food, there was no change whatever in the serum cholesterol level. With larger intakes (10 to 18 gm.) of choline-deficient food, a slight increase was occasionally observed. We would like to be able to say that regardless of the amount of choline-deficient food given, there was no increase in the serum cholesterol level. However, that may not be strictly true.

When similar rats with fatty livers and low serum cholesterol levels were given the identical food (a purified diet with about 16 per cent protein to which choline chloride had been added), the concentration of total cholesterol always rose promptly to within the normal range. Sometimes it took nearly twenty-four hours before the maximal value was reached but often it occurred within one to three hours. After remaining in the normal range for some hours, the values fell back slowly over several days. These "normalizing" effects of dietary choline were observed in rats fed a diet containing no cholesterol, hence the effect cannot in this case be due to alteration in the *absorption* of dietary cholesterol. These data suggest that one way in which choline can influence serum cholesterol levels is by affecting liver function, probably through *mobilization* of the cholesteryl esters piled up in the liver.

We have determined the changes, with time, of the concentration of total cholesterol in the serum of normal rats (males, weighing 400 gm.) following a single feeding of purified diets containing 1 per cent cholesterol, one diet containing 0.8 per cent choline chloride and one without choline. The animals ate about 25 gm. of food, which would provide about 250 mg. of cholesterol and 200 mg. choline chloride. With choline present, increases of 40 to 80 mg. per 100 ml. were observed, the maximal values occurring between one and five hours after removal of food; the values were usually back to normal within twenty-four hours. In the absence of choline, the increases in serum cholesterol were small (5 to 15 mg. per 100 ml.). The effects observed here could be due to either absorption or mobilization. The effect on mobilization seems to be limited, however, because dietary choline leads to values significantly above normal only when the diet contains cholesterol.

REFERENCES

1. Sperry, W. M. and Webb, M. A revision of the Schoenheimer-Sperry method for cholesterol determination. *J. Biol. Chem.*, 187: 97, 1950.
2. Ridout, J. H., Patterson, J. M., Lucas, C. C. and Best, C. H. Effects of lipotropic substances on the cholesterol content of the serum of rats. *Biochem. J.*, 58: 306, 1954.
3. Zlatkis, A., Zak, B. and Boyle, A. J. A new method for the direct determination of serum cholesterol. *J. Lab. & Clin. Med.*, 41: 486, 1953.
4. Zak, B., Dickenman, R. C., White, E. G., Burnett, H. and Cherney, P. J. Rapid estimation of free and total cholesterol. *Am. J. Clin. Path.*, 24: 1307, 1954.
5. Best, C. H., Lucas, C. C., Patterson, J. M. and Ridout, J. H. Effects of different dietary fats and of choline on hepatic and serum lipids of rats. *Canad. J. Biochem. & Physiol.*, 36: 613, 1958.

DISCUSSION

Dr. Robert E. Olson (*Pittsburgh, Pennsylvania*): How about more prolonged fasting? Does it elevate the cholesterol level?

Dr. Lucas: The only time that we see the values going above normal is when there is choline and cholesterol in the diet or betaine and cholesterol, but even in these cases it comes back rather promptly.

It occurred to us that if choline did cause a rise above normal in certain situations that possibly one reason that would account for the alleged difference between animal fats and vegetable fats (one being supposedly more lethal than the other in causing atherosclerosis) was that it might have something to do with the height of this peak. We made a comparison between lard and corn oil under these circumstances. The ration contained 25 per cent of the fat (lard or corn oil), 2 per cent of cholesterol, 0.3 per cent of choline chloride, and the protein level was 16 per cent.

These diets were fed to groups of fifteen rats for three weeks and then samples of blood were taken terminally. The first experiment was carried out using the Schonheimer-Sperry-Webb procedure. We took samples from different groups of rats at four, eight and twenty-four hours after the removal of food. We were not really comparing the same rats, but what we hoped were comparable rats.

We could see no difference whatever between the two groups. We were not entirely happy with the results, however, since we were dealing with different rats. More recently, after becoming familiar with the Zak method, we took sequential samples of blood from the tail vein of the same rat at one, two, four, eight, twelve, twenty-four and forty-eight hours. At no time was there a significant difference between the serum cholesterol levels of the rats fed corn oil from those fed lard. This group had been kept on the diet for three weeks.

Dr. George F. Wilgram (*Toronto, Canada*): It may be important to mention that the rats, prior to the taking of the blood, were starved for twenty-four hours. They were hungry and when food was given they all gobbled it up and were more or less in the same state of fat absorption.