THE RELATIONSHIP OF TISSUE FORMATION AND DESTRUCTION TO THE SERUM PROTEIN-BOUND CARBOHYDRATE LEVELS IN EXPERIMENTAL INFLAMMATION

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DISAGREEMENT exists as to whether an elevation of serum total protein-bound carbohydrate depends on the formation or on the destruction of tissue. From a study of chronic inflammatory and malignant neoplastic states in man Seibert, Seibert, Atno and Campbell (1947) suggested that tissue destruction accounted for this rise, whereas Shetlar, Foster, Kelly, Shetlar, Bryan and Everett (1949) held the opposite view, namely that increased carbohydrate concentration could more reasonably be correlated with tissue proliferation. The serum carbohydrate levels have also been followed in dogs with experimental inflammatory lesions, the rise in concentration being correlated with tissue proliferation or repair (Shetlar, Bryan, Foster, Shetlar and Everett, 1949). In rats bearing the Walker 256 carcinoma Shetlar, Erwin and Everett (1950) related the increase in the serum protein-bound carbohydrate level to increasing size of the tumour, which Shetlar (1952) interpreted as a form of tissue proliferation.

The conclusions drawn from these investigations depend on correlation of the serum carbohydrate levels either with the clinical state of the patient or with the gross appearances of the experimentally induced conditions. Such procedures are unsatisfactory bases of assessment, since tissue formation and destruction commonly coexist in varying proportions both in inflammatory and in neoplastic states. Microscopical study is necessary to determine the exact types of change and their relative extent at different times. We have therefore reinvestigated the relationship of tissue formation and destruction to changes in the serum protein-bound carbohydrate levels in an experimentally induced inflammatory process. Particulate silica, given intraperitoneally, was adopted as the inflammatory stimulus on account of its well known fibrogenic capacity and because an acute reaction with necrosis is prominent in the early stages. Since cortisone suppresses fibrosis, *i.e.*, one kind of tissue formation, its use seemed a suitable means of aiding the isolation of the destructive element in the inflammatory process. The results indicate that elevation of serum protein-bound carbohydrate levels is connected with the acute phase of the inflammatory reaction and not with tissue formation as judged by fibrosis. A summary of these findings has already been published (Keyser and Heppleston, 1955).

METHODS

The rabbits were young adult males of mixed breed and weighing approximately 2500 g. Each animal received, by intraperitoneal injection without anaesthesia, 2 g. of Colin Stewart silica (particle size below 7 μ) suspended in 10 ml. physiological saline. Prior to administration the suspension was sterilized in an autoclave and then agitated in a Kahn shaker for

2 hr. Three-ml. samples of blood for biochemical analysis were collected from the ear at intervals. Repeated withdrawal of large amounts of blood was avoided as this might have caused haemodilution. All blood samples were taken after the rabbits had fasted for several hours. As a check on possible alterations in blood volume, blood for haemoglobin estimation (alkaline haematin method) was taken immediately before each blood sample collected for carbohydrate determination. In the week prior to starting the injections haemoglobin and carbohydrate determinations were performed on at least two occasions on every rabbit; each animal thus served as its own basis of interpretation.

Serial estimations of carbohydrate

Changes in the serum protein-bound carbohydrate levels were followed serially in 14 rabbits. In addition to the silica, 8 of these received cortisone acetate (Roussel) subcutaneously, 25 mg. being given on the day before the injection of silica, followed by 12·5 mg. daily thereafter. Most animals were killed after 5–6 weeks but a few (including animals receiving silica alone and with cortisone) were allowed to survive for several months. The general health remained good, although some rabbits on cortisone lost weight.

Terminal estimations of carbohydrate

To secure histological correlation a further experiment was necessary. Animals were killed at appropriate intervals, as indicated by the first experiment, and a single 20-ml. fasting sample of blood was taken from each rabbit just before killing. Twelve rabbits were used, six being given cortisone as before. At intervals up to 15 days animals were killed in pairs, one of each pair having had cortisone. There was no significant change in the weight of these rabbits.

Control experiments

To ensure that the observed variations in serum protein-bound carbohydrate concentration were not attributable simply to the administration of cortisone or saline, control experiments were carried out. Three rabbits received cortisone alone subcutaneously in similar dosage to previous experiments and three others 10 ml. sterile physiological saline by intraperitoneal injection. The carbohydrate and haemoglobin levels were followed by serial estimations for 2–3 weeks.

Histological procedures

Immediate autopsy was performed on all animals and the size and extent of the peritoneal lesions were noted as well as their character. Representative lesions from the gut, from the abdominal wall or diaphragm, and from the omentum or mesentery, together with any free-lying masses of dust, were fixed in neutral formol saline and embedded in paraffin. Sections were stained by haematoxylin-eosin and for reticulin fibres. Toluidine blue and alcian blue were used to study tissue polysaccharides and the effect of preliminary incubation for 3 hr. at 37° with hyaluronidase ("Hyalase", Benger) on staining reactions with both methods was determined. The periodic acid-Schiff reaction was also applied. The Feulgen reaction was employed to demonstrate the remains of nuclear material.

These histological observations were supplemented by others obtained from preliminary experiments on 6 rabbits in which 200 mg. quartz (particle size below 5 μ) in suspension was given intraperitoneally together with cortisone subcutaneously in some of them. Though producing characteristic lesions this amount of silica did not provide an adequate rise in serum protein-bound carbohydrate concentration.

Biochemical procedures

The chemical procedures employed have been described previously (Keyser, 1954), serum total protein being separated by alcoholic precipitation, albumin with 26 per cent Na_2SO_4, and mucoprotein by the method of Winzler, Devor, Mehl and Smyth (1948). The carbohydrate was estimated by the orcinol method essentially as described by Rimington (1940).

It is known that filtrates obtained by fractionation of serum with 26 per cent Na_2SO_4 may contain small amounts of both α- and β-globulins (Jager, Schwartz, Smith, Nickerson

and Brown, 1950). On this account and also to distinguish the carbohydrate components of the different serum proteins, an attempt was made to apply the quantitative method of Köiw and Grönwall (1952). However, in the rabbit the carbohydrate bands corresponding to the globulin fractions were not sufficiently well separated to permit accurate quantitative measurements, as was also noted by Sohar, Bossak and Adlersberg (1955). Furthermore, the amount of albumin carbohydrate was so small that minor variations could not be detected. This method was therefore abandoned.

RESULTS

Serum Protein-bound Carbohydrate

The normal initial values found are given in Table I.

TABLE I.—*Normal Serum Protein-bound Carbohydrate Values*

	Number of rabbits	Number of estimations	mg. hexose/ 100 ml. (mean)	S.D.
Total protein-bound carbohydrate	33	71	92	10
Albumin-bound carbohydrate	32	68	12	2·5
Mucoprotein-bound carbohydrate	8	19	10	2·5

Since the object was to determine the relationship between the histological features of the inflammatory process and the serum protein-bound carbohydrate levels, it is considered sufficient to present the changes in these levels graphically. To compare the experimental and control groups, all carbohydrate determinations in each rabbit have been expressed as a percentage of the average normal value for that animal. Values from more than one rabbit were usually obtained at the same period of the experiment and the average was entered in the graphs. As the same trend was evident in experiments involving both serial and terminal estimations of carbohydrate, the results were combined in the graphs. It was impracticable to perform blood volume estimations but adjustment of the carbohydrate levels in proportion to variations in the haemoglobin concentration (the base-line being the average normal concentration measured before the experiment) did not affect the shape of the graphs. It is therefore unlikely that the results were complicated by haemodilution or haemoconcentration. Calculation of the total protein- and albumin-bound carbohydrate as a percentage of total protein was essentially without effect on the shape of these graphs.

Total Protein-bound Carbohydrate

After the injection of silica there was a sharp rise in the total protein-bound carbohydrate (T.P.C.), which reached a maximum in 1–3 days (Fig. 1). This rise was followed by a slower fall, the concentration of carbohydrate approaching the base-line level by about the 10th day. In animals receiving silica alone the carbohydrate content of the serum total protein rose from a mean of 1·47 per cent, to a peak of 2·29 per cent on the second day. In animals receiving silica and cortisone the corresponding values were 1·57 per cent and 2·15 per cent, the peak value again occurring on the second day. Cortisone, therefore, did not prevent the rise in T.P.C. induced by silica. Control injection of saline intraperitoneally had little influence on the carbohydrate levels. The slight elevation of

T.P.C. on the first and second day after cortisone alone rapidly subsided. The high value after 43 days on cortisone may be unrelated to the drug, since this reading is the average of estimations on only two rabbits. One of these gave a value that was little elevated, while the other rabbit had a particularly high value and showed liver necrosis and a fungus infection when killed at 123 days.

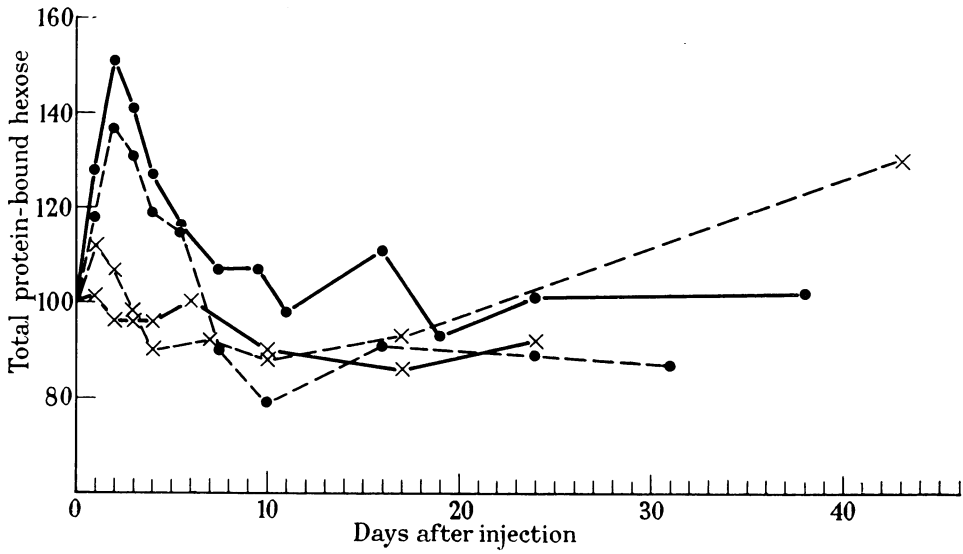


FIG. 1.—Serum total protein-bound carbohydrate (hexose), expressed as a percentage of base-line value, after silica alone (●————●), silica + cortisone (●- - - -)●, saline alone (×————×), and cortisone alone (× - - - - ×).

Albumin-bound Carbohydrate

The curves for albumin-bound carbohydrate (A.C.) resemble those obtained for T.P.C., but the fall of the levels in the silica + cortisone group was more protracted (Fig. 2). We are unable to account for the raised values found on the 10th and 17th days after cortisone alone. Expressed as a percentage of serum total protein, the albumin-bound hexose rose from a mean of 0·20 per cent to a peak of 0·40 per cent on the second day after the injection in animals receiving silica alone. In the silica + cortisone group the corresponding figures were 0·20 and 0·41 per cent, the peak value again being found on the second day. In one animal from each group, serial determinations of albumin nitrogen were made and the levels remained almost constant although the A.C. showed the usual rise.

Mucoprotein-bound Carbohydrate

In rabbits on which terminal carbohydrate estimations were made, large samples of blood were available and permitted the estimation of absolute mucoprotein-bound carbohydrate (M.C.) levels in addition to T.P.C. and A.C. (Table II). As far as can be judged from single observations on different animals at different times and over a short period, the peak level of M.C. occurred at about the same time as with the T.P.C. and A.C. levels.

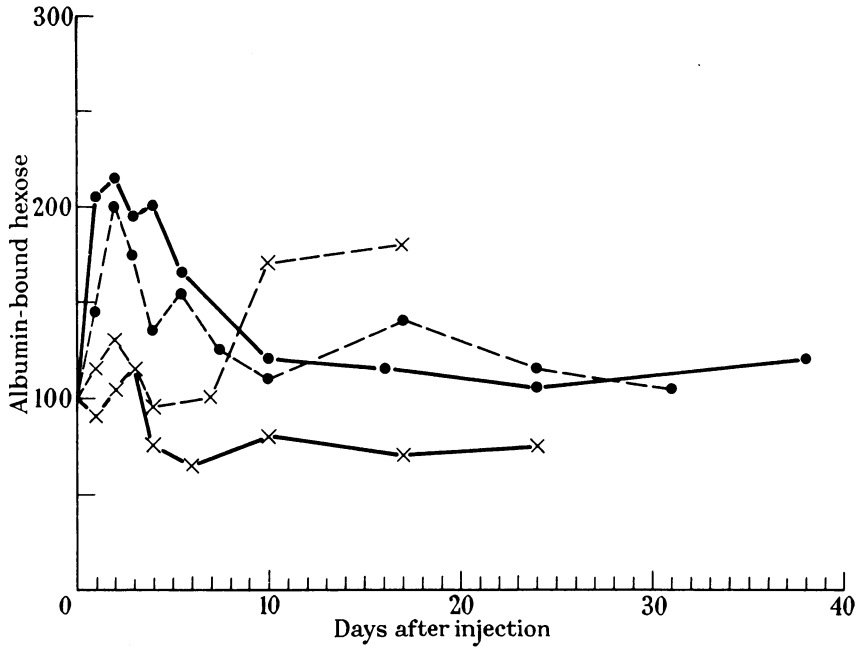


FIG. 2.—Serum albumin-bound carbohydrate (hexose), expressed as a percentage of baseline value, after silica alone (●————●), silica + cortisone (● - - - - ●), saline alone (×————×), and cortisone alone (× - - - - ×).

TABLE II.—*Serum Mucoprotein-bound Carbohydrate*

Days	mg. hexose/100 ml. After silica alone	After silica + cortisone
1	16·5	10
2	16	25
3	15	14·5
5	10·5	13
8	15	17
15	10	13·5

For normal range of values see Table I. Each value is from a different rabbit.

Histological Correlation

The inflammatory stimulus applied to the rabbits was uniform, since each was injected with the same amount of silica. Furthermore, the distribution and size of the resulting lesions in the first few days of the experiment were similar in animals whether or not they received cortisone. Later in the experiment the size of the lesions was influenced by differences in the degree of cellular proliferation and fibrosis in the two groups.

A quantitative assessment of the main characteristics of the inflammatory response at different stages is given in Table III. The various features are not always present to the same degree in lesions from different sites in the same animal. Lesions from the omentum and mesentery tended to show the more severe changes and free-lying dust masses (cortisone-treated rabbits) the least

TABLE III.—*Characteristics of the Inflammatory Response*

Experiment				Dust			
Duration in days	Type	Polymorph exudation	Cellular necrosis	Free	In macrophages	Fibroblasts	Fibres
2–3	S	+++	+++	++++	Minimal	+++	++
	S and C	+++	+++	++++	,,	+	+
7–8	S	++	++	+++	+	++++	++++
	S and C	+ to ++	++	++++	Minimal	+ to ++	+ to ++
3–5	S	+	+	Minimal to +	++++ to +++	+++	++++
	S and C	+	+	+++	+	+	++

S = Silica intraperitoneally.
C = Cortisone subcutaneously.

severe. In view of this the features of all lesions were graded on three separate occasions without reference to each other and good agreement was reached. Account has been taken of these differences in assessing the response of individual animals and an overall grading was then made for each experimental group. Polymorph exudation implies the presence of well preserved cells. Cellular necrosis, judged largely by the Feulgen reaction for nuclear deoxyribonucleic acid, mainly concerns polymorphs, but macrophages almost certainly comprise a proportion of the necrotic cells. In the lesions from some rabbits, reticulin fibres exist in masses of dust devoid of demonstrable cellular elements. Since cells are apparently concerned in the formation of reticulin it seems that fibroblasts too share in the cellular necrosis. The characteristics of representative lesions are illustrated in Fig. 3–6.

Wherever fibroblasts were actively proliferating the ground substance in the lesions showed toluidine blue metachromasia and stained with alcian blue, both reactions being totally or largely abolished by previous incubation with hyaluronidase. The ground substance did not give a positive periodic acid-Schiff reaction. These findings indicate the presence in the ground substance of a mucopolysaccharide of acidic nature (Pearse, 1953 ; Braden, 1955).

Two to three days after the injection of silica without cortisone, the inflammatory reaction includes the presence of numerous polymorphs, many being well preserved though many are necrotic, but there is little dust phagocytosis. Fibroblastic proliferation is well developed, reticulin fibres are being formed and acid mucopolysaccharide occurs in the associated ground substance. The rapid rise in serum T.P.C. and A.C. closely paralleled this early acute inflammation.

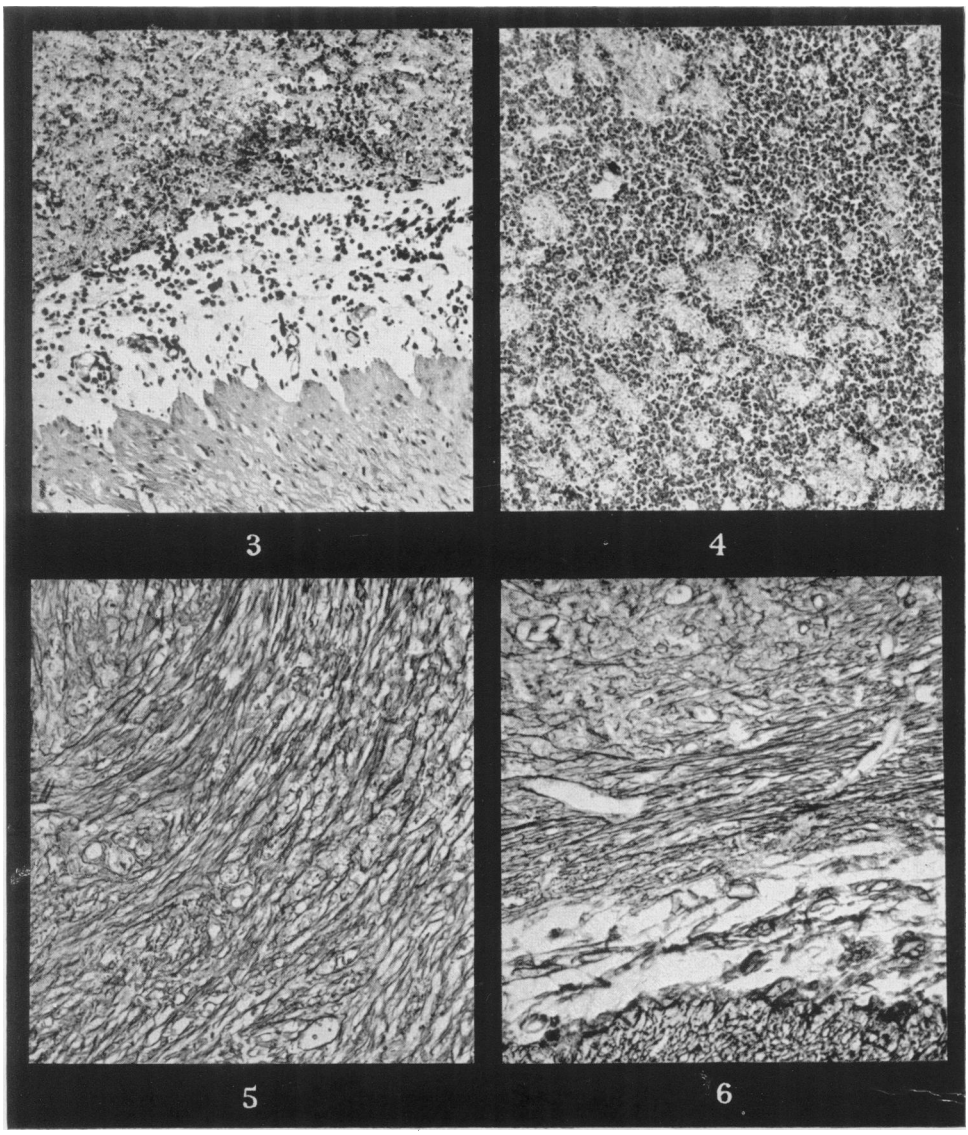
EXPLANATION OF PLATE

FIG. 3.—Silica alone. Margin of a 2-day-old lesion on the peritoneal surface of the gut. Numerous polymorphs around and in the mass of dust (seen above). Haematoxylin-eosin. × 130. Similar changes were seen in the silica + cortisone group at the corresponding period.

FIG. 4.—Silica alone. Centre of a 3-day-old lesion on the gut showing numerous necrotic polymorphs in the dust. H. & E. × 130. Similar changes were seen in the silica + cortisone group at the corresponding period.

FIG. 5.—Silica alone. Approximately a quarter of an 8-day-old lesion from the gut showing dense reticulin fibres between dust phagocytes. × 130.

FIG. 6.—Silica + cortisone. 8-day-old lesion from the gut (seen below) showing the full extent of the reticulin fibrosis. × 130.



Heppleston and Keyser.

At 7–8 days the silica lesions show increased fibroblastic activity and fibre formation, while dust phagocytosis is now more conspicuous. The T.P.C. and A.C. levels, however, are falling and at the same time polymorph exudation and cellular necrosis are less evident, as is mucopolysaccharide in the ground substance. Several weeks after injection of silica, phagocytosis is greatly increased and fibrosis, though not now so active, is more extensive and more collagenous. Acute inflammation and necrosis are still further diminished, mucopolysaccharide in the ground substance is in general scanty and the carbohydrate levels remain approximately normal.

Cortisone is without appreciable effect on polymorph exudation and cellular necrosis, but at all stages it diminishes considerably the proliferation of fibroblasts and fibre formation as well as macrophagocytosis. Mucopolysaccharide in the ground substance is reduced in proportion to the depression of fibroblastic activity. It must be added that each of the rabbits receiving cortisone showed the usual enlargement and increased glycogen content of the liver (Constantinides, Szasz and Durrach, 1955; Businco, 1954; Schmid-Bircher, 1954; Williams and Lowe, 1954). Cortisone prevents neither the early rise nor the subsequent fall in the levels of T.P.C., A.C. or M.C. Although the graphs might suggest that cortisone slightly inhibits the rise in T.P.C. and A.C. following the injection of silica, there was in fact considerable overlap between the silica and silica + cortisone groups. The difference does not therefore appear to be significant, but an experiment involving many more animals would be needed to elucidate this point statistically.

An incidental finding in rabbits receiving silica and cortisone for over 30 days was a ragged gelatinous exudate on the peritoneal surface of the abdominal wall and adjacent gut, resembling that observed by Magarey and Gough (1952). This exudate was widespread and not confined to the vicinity of silica aggregates. Histologically, it consisted of thin-walled blood vessels with scattered histiocytes and fibroblasts lying in ground substance, which in most animals stained patchily with alcian blue. This reaction was abolished by prior incubation with hyaluronidase, indicating the presence of acid mucopolysaccharide. The non-reacting areas in the ground substance gave the impression of spaces from which material had been dissolved. At the base of the gelatinous exudate in rabbits given cortisone for over 90 days fibroblastic proliferation and fibre formation occurred and were associated with toluidine blue metachromasia (abolished by hyaluronidase) in the ground substance. The most abundant gelatinous exudate was seen in a control rabbit on cortisone alone for 123 days. This animal also had liver necrosis and a fungus infection (probably aspergillosis) of the pleura and large gut. A feature common to all the rabbits showing the exudate was sarcosporidiosis affecting the muscle fibres of the abdominal wall. In the present experiments none of the rabbits receiving only silica developed the gelatinous exudate and none showed sarcosporidia. To establish an association between cortisone and the parasite in the production of this peculiar exudate, more observations are needed on the effect of cortisone on rabbits with and without sarcosporidiosis.

DISCUSSION

The results suggest that the rise in serum T.P.C. and A.C. levels is related to the early acute inflammation induced by silica. The rise cannot be correlated

with tissue formation as judged by the fibroblastic and fibrogenic responses, nor with macrophagocytosis, since these elements become prominent when carbohydrate levels are falling. The selective modifications effected in the inflammatory response by cortisone support these conclusions. The acute inflammatory reaction to silica still occurs when the serum carbohydrate concentration increases and the fall follows the same general course despite partial inhibition of the productive components of the inflammatory process. The parts played by local factors, such as cellular necrosis and depolymerization of ground substance, in the elevation of serum carbohydrate remain to be determined. The amount of mucopolysaccharide demonstrated in the ground substance of the lesions is related to fibrogenesis but not apparently to the concentration of serum protein-bound carbohydrate. Attention might also be directed to the functional activity of the liver, since it normally contributes much, if not all, of the glycoproteins and mucoproteins in the serum (Werner, 1949 ; Miller and Bale, 1954 ; Miller, 1955).

The present findings therefore do not support the conclusion of Shetlar, Bryan *et al.* (1949) that in experimental inflammation tissue formation is responsible for the rise in serum total protein-bound carbohydrate. Our results are also opposed to Shetlar's (1952) view that the rise in concentration of serum albumin-bound carbohydrate depends on tissue proliferation. We are not yet in a position to assess the part played by tissue destruction. It may be mentioned that, in patients with various forms of muscular atrophy and dystrophy, not only may the T.P.C. and M.C. levels be increased but also the level of A.C. (Keyser, Jenkins, Lloyd, Pallis and Sanders, unpublished). In such diseases muscle wasting is associated with fat replacement but not with fibroblastic proliferation, and such increase of fibrous tissue as occurs may well represent condensation of pre-existing connective tissue rather than new formation.

SUMMARY

Elevation of the serum protein-bound (total and albumin) carbohydrate closely paralleled the early acute inflammation induced in the peritoneal cavity of rabbits by silica and the levels fell when silicotic fibrosis and macrophage response became prominent.

Cortisone did not materially affect the rise or fall in concentration of carbohydrate, and the acute inflammatory reaction did not appear to be significantly modified ; fibrosis and macrophagocytosis were, however, considerably inhibited.

In these experiments, therefore, increase in serum protein-bound carbohydrate concentration can be correlated with acute inflammation but not with tissue formation as judged by fibrosis or macrophage response.

The factors responsible for the rise in carbohydrate levels remain to be determined.

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