Biochem. J. (1967) 102, 767

Studies of the Chemical Composition of a Healing Skin Wound in Rats, and of the Concentrations of Some Constituents of Tissues Distant from the Healing Wound

By JOHN CANDLISH and N. CHANDRA
Department of Biochemistry, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

(*Received* 8 *June* 1966)

1. A skin lesion was made in rats by dorsal incision and the insertion of a polythene tube. 2. Over a period of 25 days after wounding, assays were performed for ascorbic acid, DNA, hydroxyproline, methionine, tryptophan, tyrosine and free amino acids in the lesion tissue. 3. The neutral-salt-soluble proteins of the lesion tissue were fractionated on DEAE-Sephadex, with the separation of fibrinogen and γ-globulin from a serum protein fraction. 4. Over a period of 20 days after wounding, in wounded rats and in controls, assays were conducted for: ascorbic acid in lens and liver, hydroxyproline, soluble protein, methionine and water in muscle and tendon, and free amino acids in muscle. 5. Relative to controls there was a decrease in lens and liver ascorbic acid, a rise in tendon hydroxyproline, a rise in muscle free amino acids, a fall in muscle protein and a rise in tendon and muscle water.

The study of the biochemistry of wounded animals was given a primary impetus by the observation of Cuthbertson (1930) that injury is followed by an excretion of nitrogen greater than that predictable from local tissue destruction. It immediately follows that the study of wound healing cannot be confined to the wound itself, but must take into account the possible interaction of the traumatic state with all other systems in the body. One elementary way of gathering information on this is to measure the concentrations of various tissue constituents in wounded animals. Results from such measurements may be expected to point to some of those biochemical systems that are affected by wounding, and suggest further experiments to determine the causes of the alterations, if any. It is obvious that such studies will not indicate situations where turnover of components is either speeded up or slowed down (accelerated synthesis and breakdown or decreased synthesis and breakdown) so that the actual tissue concentration remains constant.

In the present work some tissue constituents thought to be of interest were assayed in wounded and control animals. This was done in conjunction with a study on the analysis of the lesion itself. The morphological and histological picture of lesion healing has been carefully built up over decades by numerous workers and expounded in standard texts. Less is known about wound biochemistry, although the considerable effort in this direction has led to a large body of information, the subject of several recent reviews (Jackson, 1964; Levenson *et al.* 1964; Viljanto, 1964). The work now described is concerned with the analysis of a skin lesion in an effort to contribute to the body of biochemical results that must be obtained to complement knowledge established by the older techniques.

EXPERIMENTAL

For each experiment, actively growing albino rats (bred in this Department) were arranged in groups of four. For the experiments involving comparison of tissue composition, the groups of four were arranged in pairs having as near as possible the same average weight, one group of four in each pair being tested with the lesion and one being the control. A separate group of four was killed on the same day as the infliction of the wound on the test animals. The experimental wound was a 6cm. incision through the dorsal skin and panniculus along the line of the vertebral column. As an extra irritant a 3cm.-long polythene tube (external diam. 6mm., internal diam. 4mm.) was buried in the wound cavity and closure effected by five or six Michel clips. Subsequently one group of wounded animals and one group of control animals were killed on selected days. In the experiments involving the comparison of tissue composition each group of four, owing to supply difficulties, was composed of two male and two female rats, all weighing 130–140g. In one experiment (indicated in the text) only females were used. Growth rates were significantly lower in the wounded rats only up to the fourth day after wounding. Thereafter there was no

difference between tests and controls. After the animals were killed, tissues were taken as required and weighed before any loss of moisture. In all these experiments tendon was exclusively from the tail, and muscle was mixed muscle from the thigh, carefully dissected free of white connective tissue.

In the experiments on the lesion itself, groups of four rats were then killed on selected days. The sutures were cut and the wound was ruptured. All tissue adhering to the edges of the cut skin and surrounding the polythene tube was then scraped or cut away. The incision itself had effectively healed after 7 days so that the new tissue could not be mechanically distinguished from the old. After this time only the lesion tissue that surrounded and invaded the polythene tube was used. The excised tissues were pooled and treated as described below.

Tissues were hydrolysed by boiling the samples with ten times their weight of constant-boiling HCl for 18hr. Hydroxyproline was assayed in hydrolysates by the Leach (1960) modification of the method of Neumann & Logan (1950). Methionine, tryptophan and tyrosine were measured on a single sample of hydrolysate (for the lesion tissue) by the method of Genevois & Barand (1956), as described by Greenstein & Winitz (1961). For muscle and tendon, methionine alone was assayed. Ascorbic acid was measured in lens and liver by the method of Roe (1954). For the lesion tissue, ascorbic acid and DNA were measured in single samples as follows. The weighed lesion tissue was ground in a mortar with acid-washed sand and a little 0·15 M-NaCl, after which 4ml. of cold 6% (w/v) trichloroacetic acid was added and used to wash the ground tissue into a 10ml. centrifuge tube. After centrifugation, three more 4ml. portions of trichloroacetic acid were used to extract the ascorbic acid, the extracts were made up to 25ml. and the ascorbic acid was assayed by the method of Roe (1954). The extracted residue was then assayed for DNA by the method of Ceriotti (1952).

The free amino acid fraction of muscle and of lesion tissue was assayed as follows. (There appeared to be no measurable quantity of free amino acids in tendon.) The weighed tissues were minced finely with scissors and then extracted three times with 3ml. portions of 80% (v/v) acetone, centrifuging after each addition. The extracts were made up to 10ml., and 0·5ml. portions were used for reaction with ninhydrin by the method described by Spies (1957). Leucine was used as standard and the results were calculated on the basis of the leucine colour with ninhydrin.

Soluble protein, in muscle, was estimated by grinding up the muscle with some acid-washed sand and a little 0·45 M-NaCl. The ground tissue was extracted with three 5ml. portions of 0·45 M-NaCl, and the extract made up to 100ml. with the same medium. Since the starting weight of fresh muscle was only 0·4–0·5g., this extraction procedure was sufficient to remove all the protein soluble in 0·45 M-NaCl, only a trace of Folin–Lowry-positive material being removed by the third extraction. Portions (0·5ml.) of the muscle extract were taken for Folin–Lowry estimation as described by Bailey (1962). For tendon the procedure was the same except that 0·1 N-acetic acid was used as the extraction agent and the extracts were made up to 25ml. This procedure resulted in the solubilization of most of the tendon material, the small amount of residue remaining being insoluble in 0·1 N-acetic acid.

For the fractionation of proteins from the lesion tissue, the weighed tissue, in each case from a single animal and usually weighing 0·4–0·8g., was mixed with an equal weight of 0·02 M-phosphate buffer, pH 6·6, and minced finely with scissors. The small bulk of the tissue plus buffer (about 1ml.) made conventional homogenization impracticable, but the thorough mincing with pointed scissors appeared to result in a dispersion into particles as fine as those normally obtained on homogenization. A complete extraction of all buffer-soluble protein in the lesion was not attempted; since equal weights of lesion and buffer were blended, the concentration of protein in the buffer after centrifugation was proportional to the concentration of protein in the tissue (soluble in the buffer). Then, since 0·5ml. of the extract was applied to the column in each case, the amount of material eluted would in turn be proportional to that originally in the tissue. This was all that was necessary to gain information on the amount and distribution of proteins at the various stages of healing. After centrifugation, 0·5ml. of the extract was applied to a column (1cm. × 25cm.) of DEAE-Sephadex A-50. This had been previously equilibrated with 0·02 M-phosphate buffer, pH 6·6. A stepwise elution procedure was adopted, as follows: 1, equilibrating buffer (50ml.); 2, 0·03 M-phosphate buffer, pH 6·6 (40ml.); 3, 0·03 M-phosphate buffer, pH 6·0 (30ml.); 4, 0·03 M-phosphate buffer, pH 5·5 (to completion of run).

Protein in the 3ml. cuts was estimated by the Folin–Lowry method (Bailey, 1962). The flow rate was fixed at 30ml./hr. The identity of the fractions was explored by immunoelectrophoresis (in the LKB Produktor apparatus) and also by semi-quantitative paper chromatography. This last was particularly valuable in checking for the presence of collagen, i.e. by the presence or absence of hydroxyproline in the fractions.

The water content of tissues was found by drying the weighed tissues in a hot-air oven at 110° to constant dry weight.

In the experiments involving comparison of tissue composition in wounded and non-wounded rats, the significance of the differences between means was calculated by means of Student's t test.

RESULTS

The results of the chemical estimations on the lesion tissue are given in Fig. 1. Fig. 1(*a*) shows that maximum lesion weight occurs at 4–7 days, coinciding with the phase of maximum oedema in the acute inflammatory reaction. In Fig. 1(*b*) the concentrations of ascorbic acid and DNA show a parallelism, this being maintained by free amino acids (Fig. 1*c*). Hydroxyproline and methionine appear to build up gradually, whereas tyrosine and tryptophan present no clear-cut pattern.

The fractionation results are presented compositely in Fig. 2. At each time of sampling, three main peaks were obtained on chromatography. None of the peaks corresponded to collagen, nor was hydroxyproline found in the eluate. This was undoubtedly because of the low salt concentration used for extraction (0·2 M-phosphate), insufficient to dissolve a detectable amount of collagen from

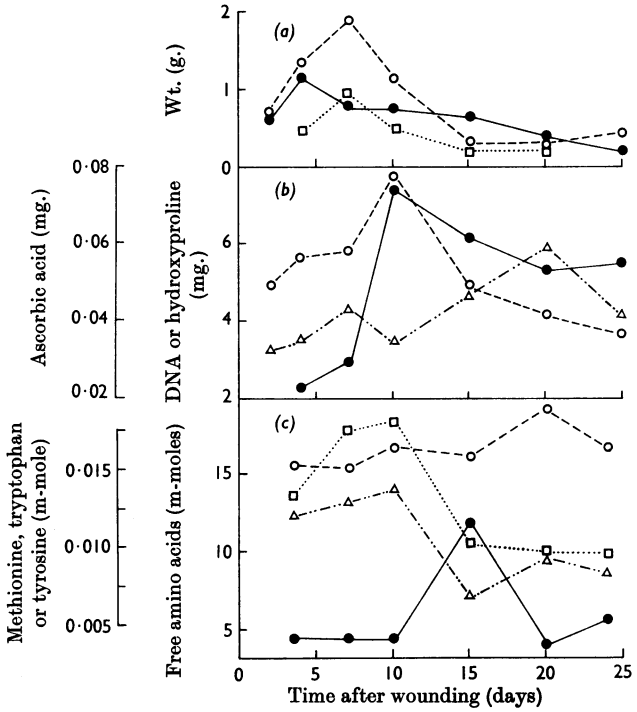

Fig. 1. Total quantities of various substances in lesion, and lesion weight, over a period of 25 days after wounding. (*a*) Weight of the lesion tissue, the results of three successive experiments (○, ● and □). (*b*) DNA (●), ascorbic acid (○) and hydroxyproline (△). (*c*) Free amino acids (□), methionine (○), tryptophan (●) and tyrosine (△).

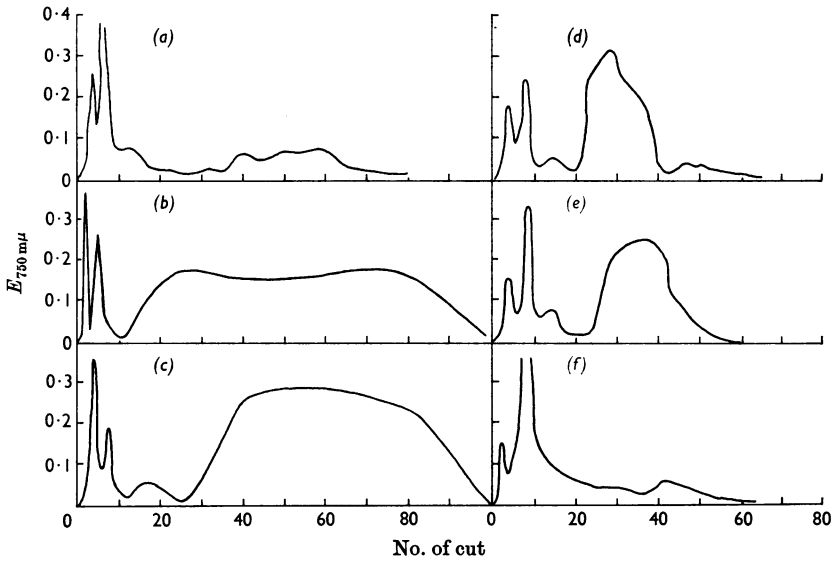

Fig. 2. Fractionation of proteins in neutral-salt extract of lesion tissue over a period of 25 days after wounding: (*a*) 2 days; (*b*) 4 days; (*c*) 7 days; (*d*) 15 days; (*e*) 20 days; (*f*) 25 days. The first peak in each elution diagram is fibrinogen, the second is γ-globulin. The small peak sometimes obtained just after γ-globulin has not been positively identified. The remaining material represents a serum protein mixture.

0·4–0·5g. of the wet lesion tissue. Neutral-salt-soluble collagen may be extracted with all ranges of ionic strength, but the efficiency of extraction increases with increasing ionic strength (Jackson & Bentley, 1960). The fractionation results therefore indicate the distribution of the plasma proteins in the inflammatory exudate at different times. The fractions were freeze-dried after the elutions were complete or concentrated by vacuum distillation and hydrolysed. When the fraction under the second peak was run against an anti-γ-globulin in the immunoelectrophoresis apparatus, a single discrete crescent was obtained. When the fractions representing the first two peaks were run against an antiplasma, one extra crescent was obtained in addition to the crescent found when they were run against an antiserum. Thus the first peak is fibrinogen. (In this way it was not necessary to prepare a specific anti-fibrinogen when antiserum and antiplasma had already been prepared.) Also, if a purified sample of fibrinogen alone was run in the column, it came off in the first 3–4 cuts. The amino acid composition of the first peak was typical of fibrinogen (high aspartic acid and glutamic acid) whereas the second peak had a very low alanine content, which is a characteristic of γ-globulin (Tristram, 1953). In some of the fractionation results (at 2, 7, 15 and 20 days in Fig. 2) there was a small peak after the γ-globulin. This has not been positively identified, owing to the very small amounts obtainable in the cuts. The remaining material, i.e. the obviously heterogeneous fraction coming off after cuts 20–25, clearly represents a mixed serum protein fraction. It had an amino acid pattern that was not typical of any specific protein.

The most striking aspect of the fractionation results is the similarity of the pattern at days 2 and 25, with the persistence of the large γ-globulin peak to the end of the observation time. The enormous increase in serum proteins at days 4 and 7 may be related to the large lesion weight and at the same time (Fig. 1*a*) be due to oedema or the inflammatory exudate.

In the experiments involving tissues distant from the lesion, Table 1 indicates that there is a significantly raised concentration of hydroxyproline in wounded animals between 7 and 10 days after wounding. A significant increase in muscle hydroxyproline was not demonstrated (Table 3). However, an examination of the differences between the means of control and test concentrations of hydroxyproline in tendon and muscles reveals a closely parallel fluctuation.

Table 2 shows that ascorbic acid is mobilized from such diverse tissues as the lens of the eye and the liver, presumably in response to the need for it in the wound. This occurs in spite of the fact that the rat is able to synthesize ascorbic acid, and it may be that in humans bearing lesions the mobilization is very drastic, especially if the diet is poor in vitamin C.

Table 2 indicates that there is a transitory rise in the concentration of free amino acids of muscle.

Table 1. *Hydroxyproline contents (mg./g.) and methionine contents (m-mole/g.) of tail tendon from wounded and control rats*

These are the results from three separate experiments, Expt. 2 consisting of female rats only. For Expt. 3 only results are expressed as mg./g. dry wt. Results are given as arithmetic means ± S.E.M.

Time after wounding (days)	Expt. no.	Control animals		Wounded animals	
		Hydroxyproline	Methionine	Hydroxyproline	Methionine
0	1	28·41 ± 3·55	—	—	—
0	2	36·18 ± 2·94	0·553 ± 0·207	—	—
0	3	99·5 ± 4·5	—	—	—
4	2	33·2 ± 1·6	0·395 ± 0·03	35·2 ± 2·22	0·472 ± 0·023
4	3	116·0 ± 4·3	—	122·2 ± 4·4	—
5	1	25·35 ± 3·6	—	31·3 ± 1·85*	—
7	2	37·7 ± 1·72	0·54 ± 0·071	44·2 ± 1·94*	0·605 ± 0·025
7	3	104·4 ± 3·1	—	120·0 ± 2·9*	—
10	1	28·1 ± 0·65	—	33·25 ± 1·75*	—
10	2	41·0 ± 1·63	0·584 ± 0·059	39·0 ± 2·85	0·388 ± 0·052
10	3	95·4 ± 3·9	—	108·3 ± 3·5*	—
15	1	31·5 ± 1·63	—	38·1 ± 6·4	—
15	2	43·5 ± 1·89	0·438 ± 0·037	—	0·492 ± 0·68
15	3	106·1 ± 3·3	—	113 ± 2·4	—
20	2	—	0·433 ± 0·045	—	0·427 ± 0·039

* Significantly different from control ($P < 0·05$).

Table 2. *Ascorbic acid (μg./g. wet wt. of tissue) in lens of eye and in liver of wounded and unwounded animals*

Results are given as arithmetic means ± S.E.M.

Time after wounding (days)	Control animals		Wounded animals	
	Liver	Lens	Liver	Lens
0	0·965 ± 0·11	4·14 ± 0·65	—	—
4	0·886 ± 0·078	—	0·539 ± 0·05*	—
5	—	3·1 ± 0·55	—	2·45 ± 0·1
7	0·756 ± 0·088	—	0·445 ± 0·16	—
10	0·91 ± 0·14	4·21 ± 0·09	0·254 ± 0·058*	2·92 ± 0·32*
15	0·8 ± 0·16	3·7 ± 0·31	0·72 ± 0·17	2·87 ± 0·12

* Significantly different from control ($P < 0·05$).

Table 3. *Free amino acids (m-moles/g. wet wt.) on the basis of a leucine standard, methionine (m-mole/g. wet wt.) and hydroxyproline (mg./g. wet wt.) in muscle tissue of wounded and control rats*

Results are given as arithmetic means ± S.E.M.

Time after wounding (days)	Control animals			Wounded animals		
	Free amino acids	Methionine	Hydroxy-proline	Free amino acids	Methionine	Hydroxy-proline
0	—	0·28 ± 0·028	—	—	—	—
4	33·3 ± 0·88	0·249 ± 0·02	1·46 ± 0·25	38·7 ± 2·36	0·293 ± 0·042	1·63 ± 0·11
7	37·8 ± 1·98	0·202 ± 0·015	1·4 ± 0·065	48·5 ± 3·43*	0·263 ± 0·025	1·67 ± 1·18
10	42·7 ± 1·9	0·245 ± 0·013	1·15 ± 0·15	42·9 ± 2·31	0·214 ± 0·036	1·2 ± 0·11
15	42·6 ± 0·65	0·207 ± 0·015	1·15 ± 0·092	42·5 ± 2·0	0·210 ± 0·012	1·19 ± 0·012
20	44·25 ± 1·19	0·17 ± 0·04	—	39·9 ± 2·23	0·210 ± 0·018	—

* Significantly different from control ($P < 0·05$).

Table 4. *Soluble protein (mg./g. wet wt.) and water (%) of muscle and tendon in wounded and control rats*

Results are given as arithmetic means ± S.E.M.

Time after wounding (days)	Muscle				Tendon			
	Control animals		Wounded animals		Control animals		Wounded animals	
	Water	Protein	Water	Protein	Water	Protein	Water	Protein
0	77·1 ± 1·0	0·221 ± 0·031	—	—	63·6 ± 1·43	0·277 ± 0·061	—	—
4	76·28 ± 0·175	0·305 ± 0·0123	77·75 ± 0·42*	0·231 ± 0·023*	66·13 ± 3·28	0·21 ± 0·003	70 ± 2·8	0·222 ± 0·029
7	75·2 ± 1·7	0·316 ± 0·0098	76·62 ± 0·305	0·246 ± 0·008*	59·3 ± 1·51	0·271 ± 0·023	64·7 ± 0·74*	0·244 ± 0·029
10	75·9 ± 0·282	0·351 ± 0·0153	76·6 ± 0·47	0·199 ± 0·0127*	60·8 ± 1·9	0·325 ± 0·015	62·51 ± 0·49	0·365 ± 0·050
15	75·3 ± 0·19	0·307 ± 0·0132	75·3 ± 0·21	0·375 ± 0·0152*	60·94 ± 0·225	0·475 ± 0·05	66·35 ± 0·11*	0·385 ± 0·024
20	75·6 ± 0·17	—	75·5 ± 0·135	—	—	—	—	—

* Significantly different from control ($P < 0·05$).

This corresponds to an accumulation in the lesion itself (Fig. 1c).

The methionine content of tendon and muscle was not significantly different in wounded and non-wounded rats (Table 3).

Table 4 shows that percentage of water rises in

the tissues of wounded animals, as expected from classical work on the subject (Wilson, 1955), and similarly the expected drastic fall in muscle protein was observed (Cuthbertson, 1930).

DISCUSSION

Cmuchalova & Chvapil (1963), from consideration of the concentrations of DNA, ascorbic acid and hydroxyproline in the carrageenan granuloma, conclude that a correlation between changes in ascorbic acid and collagen indicates that an accumulation of ascorbic acid takes place at a time when maturing of the collagen structures might be expected. This points to a participation of ascorbic acid in the control of the structural stability of the fibres. The same conclusion cannot, however, be drawn from the results of the present experiment, in which a skin lesion was studied rather than the carrageenan granuloma (Fig. 1*b*).

Methionine is said to increase the rate of development of wound tissue (Williamson & Fromm, 1955). The same workers found that the sulphur amino acid/nitrogen ratio is higher in wound tissue than in normal tissue, indicating that the protein constituents are different from those in normal tissue. Since in the present experiments methionine and hydroxyproline accumulate steadily in the wound, the deposition of collagen fibres would seem to be accompanied by the production of a methionine-rich protein.

In these experiments the methionine content of tendon and muscle was not significantly different in wounded and non-wounded rats. Fromm & Nordle (1956) found that methionine was mobilized from the muscle of animals with experimental injury, and this appeared to be due to a necessity for its conversion in the liver into cysteine needed in the lesion. Williamson & Fromm (1955) had earlier shown that methionine is mobilized from the liver itself.

Free amino acids may play some role in the precipitation of collagen fibres (Candlish & Tristram, 1963), and from Fig. 1(*c*) it appears that they are at maximum concentration at 7 days, when one would expect that fibrogenesis is being initiated. Free amino acids in blood in the early post-wounding period fall and later rise (Schreier, 1962), so there is evidently some degree of mobilization of free amino acids to the wound from blood.

The persistence of γ-globulin in the lesion when other serum proteins and fibrinogen have receded is in agreement with the results of Schilling & Milch (1955). Otherwise the fractionation results reflect the accumulation of the protein-rich inflammatory exudate into the irritated area round the tube, and the reduction of oedema as the granulomatous tissue invests the tube. Thus in this respect a degree of biochemical correlation has been obtained with the established picture of wound healing.

With respect to the results of the experiments involving the comparison of the concentrations of substances in the tissues of normal and wounded rats, it was noted that there is a significantly raised concentration of hydroxyproline, and thus of collagen, in the tendons of wounded rats. It has been reported by Trnavsky (1965) that the total hydroxyproline content of a cotton-pellet granuloma is raised if a periarthritis of the hind-paw is induced by the injection of formaldehyde while the granuloma is developing. Peacock (1963) and Adamsons, Musco & Enquist (1964) found a rise in skin collagen in rats at sites distant to incisions. The influence of a wound on collagen synthesis in diverse distant connective tissues appears then to be established. It has not yet been reported whether the increased concentration of hydroxyproline is due to increased synthesis or inhibited breakdown. There are no overriding theoretical considerations to decide on one or the other.

Schreier (1962) found that in the immediate post-operative period in surgical patients there was a decrease in the amino acids in blood and later a rise. In the present work, there was a transitory rise of free amino acids in muscle, which corresponds to a rise in the lesion itself (Table 3 and Fig. 1*c*). That free amino acids in connective tissue are under hormonal control was shown by Ryan (1964), who observed a decrease in the free amino acids of skin on hydrocortisone administration.

It is clear therefore, even from these limited experiments, that the general pattern of tissue reaction to a wound in the animal body is complex. Some substances that accumulate in the wound also accumulate in other tissues (hydroxyproline in tendon, free amino acids in muscle), whereas some substances required in the wound are depleted from other tissues (ascorbic acid from liver, methionine from liver). The presence of the wound causes a massive depletion of protein from muscle and there is a dilution of peripheral tissues by water. It seems probable that the protein metabolism of wounded animals is particularly altered in a complex fashion, and much work is required to discover the humoral or other factors underlying this.

Our thanks are due to Mr J. C. Lai for excellent technical work throughout this study, to Mr Muthu for the diagrams and to Miss L. Wan for typing.

REFERENCES

Adamsons, R. J., Musco, F. & Enquist, I. F. (1964). *Surg. Gynec. Obstet.* **119**, 323.

Bailey, J. L. (1962). *Techniques in Protein Chemistry*, p. 293. Amsterdam: Elsevier Publishing Co.

Candlish, J. K. & Tristram, G. R. (1963). *Biochim. biophys. Acta*, **78**, 289.
Ceriotti, G. (1952). *J. biol. Chem.* **198**, 287.
Cmuchalova, B. & Chvapil, M. (1963). *Biochim. biophys. Acta*, **69**, 565.
Cuthbertson, D. P. (1930). *Biochem. J.* **24**, 1244.
Fromm, H. J. & Nordle, R. C. (1956). *J. biol. Chem.* **223**, 737.
Genevois, L. & Barand, J. (1956). *Chim. anal.* **38**, 87.
Greenstein, J. P. & Winitz, M. (1961). *Chemistry of the Amino Acids*, vol. 3, p. 2133. New York: John Wiley and Sons Inc.
Jackson, D. S. (1964). In *Advances in Biology of Skin*, vol. 5 (*Wound Healing*), p. 30. Ed. by Montagana, W. & Billingham, R. E. Oxford: Pergamon Press Ltd.
Jackson, D. S. & Bentley, J. P. (1960). *J. biochem. biophys. Cytol.* **37**, 7.
Leach, A. A. (1960). *Biochem. J.* **74**, 61.
Levenson, S. M., Geever, E. F., Crowley, L. V., Oates, J. F., Bernard, C. W. & Rosen, H. (1964). *Ann. Surg.* **161**, 293.
Neumann, R. E. & Logan, M. A. (1950). *J. biol. Chem.* **184**, 299.
Peacock, E. E. (1963). *J. surg. Res.* **3**, 250.
Roe, J. H. (1954). *Meth. biochem. Anal.* **1**, 115.
Ryan, W. L. (1964). *J. invest. Derm.* **43**, 121.
Schilling, J. A. & Milch, L. E. (1955). *Proc. Soc. exp. Biol., N.Y.*, **89**, 189.
Schreier, K. (1962). In *Protein Metabolism*, p. 352. Ed. by Gross, F. Berlin: Springer-Verlag.
Spies, J. R. (1957). In *Methods in Enzymology*, vol. 3, p. 467. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Tristram, G. R. (1953). In *The Proteins*, vol. 1, part A, p. 181. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.
Trnavsky, K. C. (1965). *J. Pharm., Lond.*, **17**, 261.
Viljanto, J. (1964). *Acta chirurg. scand.* (Suppl.), 333.
Williamson, M. P. & Fromm, H. J. (1955). *J. biol. Chem.* **212**, 205.
Wilson, G. M. (1955). *Lect. sci. Basis Med.* p. 182.