LXVI. LIVER NITROGEN IN ANAPHYLAXIS.

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It has been clearly demonstrated by the work of Manwaring [1910] and of Voegtlin and Bernheim [1911], that events occurring in the liver have, at least in some species, a predominant importance in producing the train of symptoms known as "anaphylactic shock." The nature of these events, and of the changes in the liver rendering it thus specifically sensitive to a previously injected antigen, has hitherto been obscure. That the events are of such a kind as to produce a hindrance to the portal circulation, has been suggested; and there is a probability that such mechanical factors play at least some part. The recent work of Hashimoto and Pick [1914], however, seemed rich in promise of explanation along other lines. According to these observers, the liver of an animal, rendered anaphylactic by a first small injection of a foreign protein, contains a largely increased store of uncoagulable nitrogenous substances, without change of its total nitrogen from the normal average. The result is an increase of the uncoagulable fraction of the total nitrogen from a normal average of about 8 % to 22 %, or even more. According to their finding, the increase becomes definite two or three days after the sensitising injection, and attains the above-mentioned maximum at about the 14th day, thereafter very gradually declining to a lower, but still supernormal ratio, which they found still maintained as late as the 68th day. They further found that the spleen played an important part, in producing and maintaining this excess of uncoagulable nitrogen in the liver of the anaphylactic animal. Sensitisation performed the day after splenectomy, or followed by splenectomy, was not accompanied by this rise in the uncoagulable nitrogen ratio. Hashimoto and Pick's experiments were made on guinea-pigs, and they point out that this accumulation of non-coagulable nitrogen in the liver has no bearing on the plain muscle sensitisation, which gives to the anaphylactic shock in this species its most characteristic features. Their results, however, if confirmed, have an interesting relation to the

anaphylactic shock seen in such animals as the dog, in which the exclusion of the liver from circulation renders the re-injection of the sensitising antigen without effect.

Hashimoto and Pick's observations were especially suggestive in that they seemed to harmonise with other observations on anaphylaxis and similar conditions. Thus it has been shown, by several workers, that the symptoms of the anaphylactic shock correspond closely with those following the injection of certain products of the partial cleavage of proteins. It has been shown also, that a second parenteral administration of a foreign protein is followed by an increased output of nitrogen in the urine, considerably greater than could be accounted for by the nitrogen-content of the injected substance. If the result of a first injection then, were, as stated, to cause an accumulation of protein cleavage-products in the liver cells, and if these were released into the circulation as the result of the second injection, a possibility would be presented of co-ordinating a number of hitherto imperfectly connected observations. With a view to examining these possibilities further, our ultimate object being a closer analysis of the process of sensitisation and anaphylactic shock in the dog, we have repeated Hashimoto and Pick's experiments; but we find ourselves held up, at the outset of the investigation, by a complete failure to confirm their observations. We are at a loss to account for the discrepancy. Our technique has differed from theirs in no essential particular. We have examined in all 13 guinea-pigs sensitised with injections of horse-serum, varying from 0.1 to 2 cc., and have not found one in which the ratio of non-coagulable to total liver-nitrogen lies outside the range of variation seen in normal controls. The experiments on controls were interspersed among the anaphylactic series, so that seasonal or dietary variation would affect both series alike. The average values in the two series are practically identical.

Being unable to confirm the fundamental observation of the presence of excess of uncoagulable nitrogen in the anaphylactic liver, we were not in a position to study the effect, on the alleged excess, of subjecting the animal to anaphylactic shock. Nor have we, save for casual observations of the rate of autolysis for a few hours at room temperature, which showed no significant difference between normal and anaphylactic liver, made any attempt to follow the further observations of Hashimoto and Pick, since these postulate the accuracy of the main observation, which we fail to confirm. Nor have we, as we had proposed, extended the observations to other species.

The publication of results, which are a mere contradiction of those

obtained by previous investigators, without the possibility or near prospect of the personal communication, which we should have desired, and which might have helped us to an explanation of the discrepancy, is at present an unfortunate necessity.

EXPERIMENTAL.

The observations were made on young, healthy guinea-pigs, varying in weight from 210 to 385 g., taken from a common stock, kept under identical conditions. All the observations were made between August 31st and October 14th and the diet (hay, oats, bran and green stuff) was not varied in composition or quantity throughout the series. Nevertheless, to exclude the influence of seasonal variation, or of any unnoticed change in diet, the observations on normal controls were interspersed among the series on anaphylactic animals.

The anaphylactic series consisted of 13 guinea-pigs, each sensitised by a subcutaneous injection of normal horse-serum; five receiving 0.1 cc., six receiving 0.5 cc., and two receiving 2 cc. each. The livers were examined at an interval varying from 11 to 21 days after the injection. One of the six animals sensitised by 0.5 cc. of horse-serum was killed on the 12th day by a second, intravenous injection of 0.5 cc. of the same serum; death took place within five minutes from typical acute shock, with extreme and permanent distension of the lungs. The others were killed by a blow on the head, and in all cases the animal was bled as completely as possible by severing the blood-vessels in the neck and compressing the abdomen. The 12 normal controls were killed in the same manner.

The pale and nearly bloodless liver was removed rapidly after killing and was immediately cut into portions of suitable size for the different determinations. In a few trial experiments on normal livers we used a method identical in all details with that employed by Hashimoto and Pick; the whole liver, after removing the gall-bladder and large vessels, was pounded in a mortar and then rubbed through fine nickel gauze, portions for estimation being weighed out from the semi-fluid pulp so produced. Such a method provided an ideal safeguard against local variations of composition. At the same time it is troublesome and wasteful—the loss in the meshes of the gauze being such as to make it difficult to obtain four adequately large samples from the liver of a small guinea-pig. Drying is also apt to occur in the thinnish film of material produced. We made a few experiments, therefore, to discover whether local variations of composition occur in the

liver of sufficient magnitude to weaken the accuracy of estimates carried out on different samples cut from a whole liver. We found that the values, whether for total or non-coagulable nitrogen, obtained from different pieces of the same liver, were remarkably concordant—showing at least as good a correspondence as those obtained from different portions of the disintegrated and mixed liver substance. The small error was clearly the error of the Kjeldahl method, and not that of imperfect sampling. We, therefore, abandoned the preliminary disintegration, and simply weighed out in clean, tared beakers as many pieces of whole liver substance as were required, merely avoiding the central portions, where large vessels and ducts are present in undue proportion to the liver cells.

For determining total nitrogen pieces of from 0.5 to 1 g. were usually taken, and these were at once washed from the beakers into Kjeldahl flasks. To each flask were added 20 cc. of strong sulphuric acid, 10 g. of potassium sulphate, and a small fragment of copper gauze. A preliminary heating on a water-bath, before burning, was found useful in diminishing the trouble of bumping.

For determinations of non-coagulable nitrogen portions of 2 to 2.5 g. were usually employed. Two different methods of coagulation were used. One of these gave, with some uniformity, slightly lower values than the other. Estimates on both normal and anaphylactic livers were made by both methods, and comparisons were made of normal and anaphylactic values obtained by the same method.

Method I. This is identical with the method employed by Hashimoto and Pick, except in that disintegration is performed after, instead of before weighing. The sample of liver is transferred with a little distilled water to a glass mortar and rubbed up into as fine a pulp as possible. More distilled water is added and rubbed up with the liver-pulp. After a brief settling the supernatant fluid is poured back into the beaker, the residue of less thoroughly ground fragments subjected to renewed rubbing, more water added and poured off; the process is repeated till nothing remains but a few fine colourless shreds. These are finally washed into the beaker and the mortar and pestle thoroughly washed with several more small quantities of distilled water, which are added to the mixture. The fine emulsion thus obtained was made up, as in Hashimoto and Pick's experiments, to about 70 cc. A pinch of salt was added and the beaker transferred to a boiling water-bath; three drops of 20 % acetic acid were then added from a fine pipette, and heating continued till coagulation was quite complete.

TABLE I.

Number and Date of Experiment	Weight of guinea-pig in g.	Volume of sensitising injection of horse serum and time since injection	Number of liver-sample	Weight of sample, g.	Determination made (total or non-coagulable nitrogen)	Method for non-coagulable nitrogen	Milligrams of nitrogen	Percentage of nitrogen	Mean	Percentage of non-coagulable in total nitrogen by Method I	Percentage of non-coagulable in total nitrogen by Method II	Percentage of non-coagulable in total after standing 3 hours
1 Aug. 31st	320	None (control)	1	1-174	Total		35-07	2-99	2-97	10		
			2	1-680	"	I	49-35	2-94				
			3	1-047	Non-coagulable	"	3-36	0-321	0-298			
			4	1-630	"	"	4-48	0-275				
2 Sept. 1st	2 guinea-pigs, each about 250 grammes. Livers mixed	None (control)	1	2-017	Total		62-37	3-07	3-12	9-2		
			2	1-753	"	I	55-65	3-17				
			3	2-709	Non-coagulable	"	7-65	0-282	0-286			
			4	3-025	"	"	8-78	0-290				
3 Sept. 3rd	280	None (control)	1	1-593	Total	II	53-55	3-36	3-36			7-7
			2	1-738	Non-coagulable	"	4-57	0-263	0-260			
			3	1-880	"	"	4-85	0-258				
4 Sept. 8th	390	None (control)	1	3-211	Total	II	94-92	2-96	2-96			7-5
			2	2-084	"	"	61-74	2-96	0-222			
			3	1-881	Non-coagulable	"	4-2	0-222				
			4	1-790	Lost by accident	"						
5 Sept. 9th	305	None (control)	1	1-363	Total		48-8	3-58	3-56			8-4
			2	1-497	"	II	53-0	3-54				
			3	1-203	Non-coagulable	"	3-7	0-30	0-30			
			4	1-522	"	"	4-6	0-30				
6 Sept. 11th	220	0-1 cc. 11 days	1	1-766	Total		62-37	3-53	3-48			7-9
			2	0-2638	" (Micro Kjeldahl)	II	9-1	3-44				
			3	1-800	Non-coagulable	"	4-95	0-275	0-274			
			4	1-792	"	"	4-90	0-273				
7 Sept. 14th	245	0-1 cc. 14 days	1	0-599	Total		19-32	3-22	3-16			7-95
			2	0-939	"	II	29-09	3-10				
			3	1-685	Non-coagulable	"	4-39	0-260	0-251			
			4	1-945	"	"	4-71	0-242				
8 Sept. 16th	209	None (control)	1	0-599	Total	I	19-22	3-21	3-21	8-3		
			2	0-760	"	"	24-36	3-21				
			3	2-567	Non-coagulable	"	6-93	0-270	0-267			
			4	2-570	"	"	6-82	0-265				

9 Sept. 16th	245	0.1 cc. 16 days	1 2 3 4	1.070 0.846 2.632 2.933	Total " Non-coagulable "	1 "	24.68 19.64 5.78 6.40	2.31 2.32 0.219 0.219	2.32 0.219	9.5
	10 Sept. 17th	None (control)	1 2 3 4	0.941 0.826 2.012 2.207	Total " Non-coagulable "	1 "	23.5 20.5 4.4 4.7	2.50 2.48 0.219 0.213	2.49 0.216	8.7
			1 2 3 4	0.928 0.657 2.849 2.347	Total " Non-coagulable "	1 "	31.7 23.3 7.1 6.2	3.42 3.55 0.250 0.265	3.48 0.258	7.4
			1 2 3 4	0.873 0.983 2.379 2.459	Total " Non-coagulable "	1 "	27.8 30.7 5.65 5.8	3.18 3.12 0.238 0.236	3.15 0.237	7.5
13 Sept. 21st	270	0.1 cc. 21 days	1 2 3 4	1.112 1.172 2.327 2.543	Total " Non-coagulable "	1 "	40.0 43.0 6.5 6.8	3.60 3.67 0.279 0.267	3.63 0.273	7.5
			1 2 3 4	1.490 1.623 3.054 2.151	Total " Non-coagulable "	1 "	45.0 50.4 8.0 5.8	3.02 3.10 0.264 0.270	3.06 0.267	8.7
			1 2 3 4	1.548 1.265 2.441 2.247	Total " Non-coagulable "	1 "	48.9 41.4 6.15 5.75	3.16 3.27 0.252 0.256	3.22 0.254	7.9
	294	0.5 cc. 13 days	1 2 3 4	1.055 1.609 2.255 2.613	Total " Non-coagulable "	1 "	41.3 61.6 6.2 7.2	3.90 3.82 0.275 0.275	3.86 0.275	7.1
17 Oct. 1st	278	None (control)	1 2 3 4	0.849 0.980 2.401 2.494	Total " Non-coagulable "	1 "	30.8 34.9 5.95 6.00	3.62 3.56 0.247 0.241	3.59 0.244	6.8
			1 2 3 4 5 6	0.978 0.951 2.871 2.464 2.077	Total " Non-coagulable " Lost by accident	1 11	23.2 22.7 6.8 5.6 4.5	2.37 2.38 0.250 0.227 0.217	2.38 0.238 0.217	10 9.1
			1 2 3 4 5 6	0.534 0.411 2.040 2.133 1.895 1.735	Total " Non-coagulable " Lost by accident	11 1 "	18.3 13.5 5.1 5.2 5.0	3.43 3.29 0.250 0.244 0.264	3.36 0.247 0.264	7.9 7.3
	285	0.5 cc. 17 days	1 2 3 4 5 6							

TABLE I *continued*

Number and Date of Experiment	Weight of guinea-pig in g.	Volume of sensitising injection of horse-serum and time since injection	Number of liver sample	Weight of sample, g.	Determination made (total or non-coagulable nitrogen)		Method for non-coagulable nitrogen	Milligrams of nitrogen	Percentage of nitrogen	Mean			Percentage of non-coagulable in total nitrogen by Method I	Percentage of non-coagulable in total nitrogen by Method II	Percentage of non-coagulable in total nitrogen after standing 3 hours
20 Oct. 7th	310	0.5 cc. 19 days	1	0.747	Total			17.92	2.40	2.38	9.4	9.1	9.4	9.1	12.2
			2	0.704	"			16.66	2.37						
			3	2.318	Non-coagulable		1	5.25	0.226						
			4	2.408	"		"	5.4	0.224						
			5	2.327	"		"	5.0	0.215						
			6	2.205	"		"	4.8	0.218						
			7	2.004	"		"	5.7	0.284						
			8	2.009	"		" (After 3½ hrs. autolysis)	6.0	0.298						
21 Oct. 8th	275	2 cc. 15 days	1	0.440	Total			16.2	3.70	3.65	8.2	7.5	8.2	7.5	
			2	0.610	"			22.0	3.61						
			3	2.244	Non-coagulable		1	6.8	0.303						
			4	2.242	"		"	6.6	0.294						
			5	1.274	"		"	3.6	0.283						
			6	1.633	"		"	4.35	0.266						
22 Oct. 8th	265	2 cc. 15 days	1	0.593	Total			21.4	3.61	3.59	10.1	8.7	10.1	8.7	
			2	0.909	"			32.3	3.57						
			3	1.751	Non-coagulable		1	6.35	0.363						
			4	2.231	"		"	8.1	0.363						
			5	1.659	"		"	5.3	0.319						
			6	1.793	"		"	5.5	0.307						
23 Oct. 9th	360	None (control)	1	0.594	Total			21.80	3.67	3.65	8.3	7.9	8.3	7.9	
			2	0.726	"			26.32	3.63						
			3	1.738	Non-coagulable		1	5.4	0.311						
			4	1.915	"		"	5.6	0.293						
			5	2.158	"		"	6.2	0.287						
			6	2.219	"		"	6.5	0.293						
24 Oct. 12th	390	None (control)	1	1.135	Total			29.43	2.60	2.64	9.2	8.3	9.2	8.3	11.2
			2	0.577	"			15.47	2.68						
			3	2.584	Non-coagulable		1	5.63	0.218						
			4	2.638	"		"	5.75	0.218						
			5	2.505	"		1	6.2	0.247						
			6	2.495	"		"	5.95	0.239						
			7	2.612	"		"	8.4	0.283						
			8	2.374	"		" (After 3 hrs. autolysis)	8.2	0.307						
25 Oct. 13th	260	None (control)	1	0.785	Total			25.97	3.31	3.27	11.3		11.3		
			2	1.155	"			37.31	3.22						
			3	2.105	Non-coagulable		1	8.2	0.389						
			4	2.137	"		"	7.45	0.349						

The time on the water-bath was in all cases about 30 minutes. The beaker and its contents were then cooled, the coagulum carefully broken up with a rod into fine fragments, and the whole, with repeated washings, transferred to a 100 cc. standard flask, the volume being then accurately adjusted with distilled water. The flask was well shaken and the contents filtered through an ordinary folded filter paper.

Method II. The piece of liver, as soon as it was weighed, was partially coagulated whole, by pouring into the beaker about 10 cc. of boiling distilled water faintly acidulated with acetic acid, heating on the water-bath for one minute and then cooling. By this preliminary coagulation the disintegration in the mortar was rendered much easier. The piece of liver was ground first with a small quantity, and then with the whole of the fluid in which it was coagulated. A fine creamy emulsion was obtained, and, by pouring off after brief settling and regrinding the residue with successive portions of distilled water, a suspension of the whole substance in a very fine state of division was obtained. The fluid was again made up to 70 cc. and definitive coagulation carried out on the same lines as in Method I. The transference of the fluid and coagulum to the standard flask was rendered easier in this case by the finely divided state of the coagulum. The volume was made up to 100 cc. and filtration carried out as in Method I.

Of the filtrate obtained by either method a convenient volume (usually 70 cc.) was taken for Kjeldahl estimation. Hashimoto and Pick stipulate that the filtrate shall be free from any trace of opalescence, though they admit, in a footnote, that opalescent filtrates do not differ materially in nitrogen-content from perfectly clear ones. They suggest that the opalescence is due to glycogen, and their suggestion is certainly correct. It may be said, indeed, that with the majority of livers, worked up with sufficient rapidity, a filtrate free from traces of opalescence is not obtainable. This is especially the case with Method II, in which the preliminary scalding effectively stops post-mortem glycogenolysis. The nitrogen value obtained by Method II is, however, regularly somewhat lower than that obtained from the same liver by Method I. It is clear, then, that this opalescence has no connection with imperfect coagulation, and we have ignored it. Glycogen-rich livers, giving opalescent filtrates, naturally show lower percentages of both total and non-coagulable nitrogen than livers which are practically nitrogen-free; but the ratio of non-coagulable to total nitrogen is not disturbed. In every experiment duplicate samples were worked up.

In several cases the non-coagulable nitrogen was estimated by both Methods I and II—two samples being worked up by each method.

The ammonia resulting from the Kjeldahl combustion was distilled into standard sulphuric acid, 10 cc. N/2 acid being used for a total, 10 cc. N/10 acid for a non-coagulable nitrogen determination. Titration was carried out with N/20 caustic potash, methyl red being used as indicator. The standards were carefully checked on several occasions during the series of experiments. The results are given in Table I.

It will be seen that Method I for determination of the non-coagulable nitrogen regularly gives a somewhat higher percentage than Method II, when both are applied to the same liver. For purposes of comparison, therefore, it is more accurate to consider separately the results obtained by the two methods, as in Table II.

TABLE II.
Percentage of Non-coagulable in Total Nitrogen.

Method I.			Method II.		
Normal	Anaphylactic	Sensitising dose and interval	Normal	Anaphylactic	Sensitising dose and interval
10.0	9.5	0.1 cc. 16 days	7.7	7.9	0.1 cc. 11 days
9.2	7.4	0.1 cc. 17 "	7.5	7.95	0.1 cc. 14 "
8.3	7.5	0.1 cc. 21 "	8.4	—	—
8.7	8.7	0.5 cc. 11 "	8.3	—	—
7.5	7.9	0.5 cc. 12 "	9.2	—	—
6.8	7.1	0.5 cc. 13 "	—	—	—
8.3	10.0	0.5 cc. 14 "	—	9.1	0.5 cc. 14 "
9.2	7.9	0.5 cc. 17 "	—	7.3	0.5 cc. 17 "
11.3	9.4	0.5 cc. 19 "	—	9.1	0.5 cc. 19 "
—	8.2	2 cc. 15 "	—	7.5	2 cc. 15 "
—	10.1	2 cc. 15 "	—	8.7	2 cc. 15 "
Average 8.8	8.5	—	8.2	8.2	—

It is sufficiently clear that, whichever method is used, there is no significant difference between the percentages obtained. We are entirely unable to explain the failure of our animals to show the striking difference exhibited by those of Hashimoto and Pick. That our guinea-pigs were satisfactorily sensitised is clear from the rapid death, in response to a second injection, of the one animal thus tested. Being unable to obtain the accumulation of non-coagulable nitrogenous substances which Hashimoto and Pick described, we are not in a position to investigate it further. Our results seem to us, however, sufficiently clear to indicate that, whatever its origin, the condition described by them had no relation to anaphylaxis.

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