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A Collaborative Study of Heparins from Different Sources*

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Until about 10 years ago heparin was prepared from ox lungs and was regarded as a homogeneous polymer (of a sulfated mucopolysaccharide) with a relatively constant biological activity of about 100 IU/mg. The mucus and mucosa of the intestine of hog, sheep and ox have now become the main industrial source of bulk starting material. Preparations from this new source have much higher specific biological activity and have been shown to consist of mixtures of components with a number of different chemical and physical characteristics. It had been variously reported that preparations of heparins from the 2 sources gave significantly different estimates when bioassayed by the 2 different pharmacopoeial assay methods which are in wide use. If this is in fact the case these discrepancies could cause trouble in commerce, national control and clinical usage.

This paper describes an international collaborative study to investigate this and other problems in the assay of heparin. In this study preparations of heparin made from the intestinal mucosa of each of 3 species (hog, sheep and ox) were biossayed with the International Standard for Heparin (from ox lung) established in 1958. Two of these preparations were national standards.

The results showed differences between the estimated relative potencies of heparins of lung and mucosal origin, but they were not so consistent as to necessitate a separate international standard for each, although the imprecision of the assays may have concealed significant heterogeneity arising from real differences between the preparations. A further problem brought to light in this study was that, with the 2 pharmacopoeial assay methods in wide use, simple replicate assays using the same substrate gave estimates so precise that an analysis of variance on them was meaningless, whereas estimates between laboratories or with different substrate batches could vary by as much as 40 %–50 %.

It is suggested that estimates of the biological activity of heparin preparations should be based on several independent assays using different batches of substrate.

The biological assay methods currently used were devised some 15–20 years ago and, in the light of modern knowledge of mechanisms of blood clotting, are regarded as unsatisfactory in a number of respects. It is hoped that research into the mechanism of action of heparin will lead to improved methods of assay.

In 1963 the WHO Expert Committee on Biological Standardization (1964a) requested the National Institute for Medical Research, London, to investigate the need for additional international standards for heparin since evidence had been obtained which suggested that the current (second) International Standard for Heparin, which was prepared from bovine lung tissue, might not be suitable to serve as a standard for the assay of heparins from the other tissues, notably intestinal mucosa, currently used as a major commercial source. It has been reported that independent assays of batches of heparin from various sources have given significantly different and sometimes unreproducible potencies when assayed by the methods described in the British Pharmacopoeia (BP) and the United States Pharmacopeia (USP).

This paper describes an international collaborative study in which preparations of heparin made from the intestinal mucosa of each of 3 species (hog, sheep, ox), and the International Standard were all compared by different assay methods, in particular,

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by that of the United States Pharmacopeia (1965) and that of the British Pharmacopoeia (1963).

In view of the possible need for an additional or a new international standard, one of the preparations of heparin of mucosal origin included in this study consisted of a large quantity of material which was sealed in ampoules under the conditions normally used for the preparation of an international standard.

MATERIALS INCLUDED IN THE STUDY

The International Standard for Heparin established in 1958

This consisted of 2000 ampoules each containing some 20 mg of heparin made from bovine lung tissue by a process that included precipitation as a barium salt. On the basis of an international collaborative study, the potency of the heparin was assigned as 130 IU/mg (Bangham & Mussett, 1959) and the standard was established by the WHO Expert Committee on Biological Standardization (1959) to replace the existing (first) International Standard for Heparin.

The USP reference standard for sodium heparin

In March 1965, a batch of 100 tablets, each weighing about 20 mg, was received from the office of the United States Pharmacopeia, New York, and these were distributed singly into ampoules at the National Institute for Medical Research, London. The material is of mucosal origin and the potency was stated to be 2.8 USP units/mg.

The British standard for heparin of mucosal origin

This consists of ampoules containing 10.6 mg ($\pm 1\%$) of freeze-dried heparin from bovine intestinal mucosa made by a process that did not include precipitation as a barium salt. On the basis of a collaborative assay in terms of the International Standard, it was assigned a potency of 1250 British units/ampoule; this figure was approximately midway between the means of estimates made by the USP (1965) and BP (1963) assay methods (Walton, Ricketts & Bangham, 1966).

Heparin from porcine intestinal mucosa

A single batch of 41.5 g of heparin was obtained from the manufacturer,[1] who supplied the following data:

Moisture	11.6%
Total nitrogen	2.11%
Total sulfur	10.5%
Total sodium	12.6%
Sulfated ash	39.8%
Specific optical rotation $(\alpha)_D^{20}$	+52.7
Anticoagulant activity—BP (1963) assay	164.0 British units/mg
Anticoagulant activity—USP (1965) assay	165.5 USP units/mg

The estimates of potency of the bulk material are given in terms of the British standard for heparin of mucosal origin.

In 1965 the bulk material was dissolved in 4.25 litres of glass-distilled water, the pH of the solution being 7.7. The heparin solution was filtered through a Millipore sterilizing membrane and distributed in approximately 1.1-ml amounts into some 3600 ampoules. The average weight of solution per ampoule was 1.0095 g with a maximum deviation of $\pm 1\%$ determined by check weighings. All the ampoules were dried as a single batch in a shelf freeze drier and, after being fitted with plastic plugs to restrict gaseous diffusion, they were stored over phosphorus pentoxide for 6 days under high vacuum in secondary desiccation chambers.

The chambers were filled with pure dry nitrogen and the ampoules removed and sealed. The ampoules were subsequently checked for structural flaws and stored at $-10°C$ in the dark.

The mean moisture content of 6 ampoules was 1.41% (measured as loss of weight after drying at 56°C over phosphorus pentoxide at high vacuum). The mean oxygen content on another 6 ampoules was 0.13%.

Heparin from sheep intestinal mucosa

This material was obtained from the manufacturer[2] and was freeze-dried in ampoules at the National Institute for Medical Research under conditions similar to those used for the heparin from porcine mucosa.

THE COLLABORATIVE STUDY

A total of 13 laboratories took part in this collaborative study. The names of the participants are given in an Annex, but elsewhere in this report they are distinguished only by a number which does not necessarily correspond with the order of listing in the Annex.

[1] Through the generosity of the Evans Biological Institute, Runcorn, Cheshire, England.

[2] Union International Ltd., London, England.

TABLE 1
DETAILS OF ASSAYS USED BY PARTICIPANTS

Lab. no.	Total no. of assays	Assay method	No. of assays	No. of materials compared in one assay
1	24	United States Pharmacopeia (1965)	24	5
2	85	United States Pharmacopeia (1965)	85	2
3	33	Clot protein	13	6
		BP + coagulometer [a]	20	3
4	26	United States Pharmacopeia (1965)	6	6
		British Pharmacopoeia (1963)	20	3
5	80	United States Pharmacopeia (1965)	62	2
		British Pharmacopoeia (1963)	18	2
6	36	Thrombin titration [b]	36	2
7	12	USP-Pritchard [c]	12	6
8	33	British Pharmacopoeia (1963)	33	2
9	12	British Pharmacopoeia (1963)	11	6
		In vivo (clotting time)	1	—
10	68	Howell [d]	51	5
		In vivo (clotting time, partial thromboplastin time and lipoprotein lipase)	13	3
		Colorimetric—DK2 [e]	2	2
		Colorimetric—Lovibond [f]	2	2
11	22	United States Pharmacopeia (1965)	10	6
		British Pharmacopoeia (1963)	12	4
12	10	British Pharmacopoeia (1963)	10	3
13	24	United States Pharmacopeia (1965)	24	4

[a] British Pharmacopoeia, 1963, using a Walton/Wright coagulometer.
[b] Kjems & Wagner (1948).
[c] United States Pharmacopeia, 1965, as modified by Pritchard (1956).
[d] Jaques & Bell (1959a).
[e] Jaques & Wollin (1967).
[f] Jaques & Bell (1959b).

The 5 materials described above were sent to participating laboratories in coded ampoules together with a second set of ampoules of the porcine mucosal heparin bearing a different code letter. In order to obtain the maximum number of comparisons from the minimum number of assays, each participant was sent a design for his assays which was based on the number of preparations that he could compare together at any one time. Participants were encouraged to use more than one assay method.

A total of 9 different biological assay methods were used. Of these the following 5 *in vitro* methods were included: the BP method (British Pharmacopoeia, 1963); Howell method (Jaques & Bell, 1959a); a method based on the measurement of clot protein and thrombin titration (Kjems & Wagner, 1948); the USP (1965) method; and a modification of the USP method (Pritchard, 1956). Two laboratories performed *in vivo assays*; Laboratory 9 measured the clotting time using unanaesthetized dogs, and Laboratory 10 measured clotting time, partial thromboplastin time and lipoprotein lipase on blood samples from the same dog (Rezansoff & Jaques, 1967). In addition, Laboratory 10 did 2 colorimetric assays (Jaques & Bell, 1959b; Jaques & Wollin, 1967). A summary of the assays carried out by each laboratory is given in Table 1.

STATISTICAL ANALYSIS

The results of the BP, clot protein and thrombin titration assays were analysed according to the usual method of analysis for parallel line assays. The clot protein assays and all the BP assays, except those

from Laboratory 4, were analysed by relating the log response to log dose. The log transformation was not sufficient to remove the correlation between the variance of the response within dosage groups for Laboratory 4 and so these assays were analysed by relating the reciprocal of the response to log dose. No transformation of the response was used for the thrombin titration assays. The regression of the log dose–response lines was found to be significant at the 1% level for all these assays but significant differences ($P<0.01$) in the slopes of the lines were observed in the analysis of a few of the assays.

Seven different comparisons between preparations have been made. The potency of the porcine mucosal heparin was estimated in terms of the International Standard, the British standard and the USP reference standard; the potencies of the duplicate set of coded ampoules of the porcine mucosal heparin and the sheep mucosal heparin were expressed in terms of ampoules of the porcine mucosal heparin as potency ratios. When, in a multiple assay, 2 differently coded ampoules of the porcine mucosal heparin were used, the responses to the doses from these 2 ampoules were combined before calculating estimates of potency in terms of the other 3 standards, or when estimating the potency of the sheep mucosal heparin. In addition, where either the British standard or the USP reference standard was included in the same assay with the International Standard, the potencies of ampoules of these materials have, in this report, been expressed in terms of the International Standard. It should be appreciated that the potencies calculated from a multiple assay are not independent since in each instance the same data for the common standard are used. Summaries of these various comparisons are given in Tables 2–8. In each of the *in vivo* assays submitted by Laboratory 10, measurements of the clotting time, partial throm-

TABLE 2

PORCINE MUCOSAL HEPARIN PREPARATION IN TERMS OF ITSELF

Assay method	Lab. no.	No. of assays	Mean potency ratio	Confidence limits (P = 0.95)
British Pharmacopoeia (1963)	3	6	1.014	0.997–1.031
	4	6	0.960	0.908–1.014
	5	3	0.998	0.964–1.033
	8	4	1.000	0.989–1.009
	9	11	1.063	1.001–1.125
	11	6	1.013	0.980–1.048
	12	3	1.008	0.987–1.029
United States Pharmacopeia (1965)	1	12	0.996	0.975–1.017
	2	8	0.975	0.932–1.020
	4	4	0.999	0.853–1.169
	5	8	0.992	0.957–1.028
	7	12	1.040	1.031–1.048
	11	10	1.008	0.973–1.043
	13	13	1.014	1.004–1.025
Clot protein	3	13	0.982	0.951–1.013
Thrombin titration	6	4	0.991	0.971–1.012
Howell	10	18	1.086	1.033–1.142
Colorimetric—DK2	10	2	1.007	—
Colorimetric—Lovibond	10	2	0.994	—
In vivo (clotting time)	10	2	1.011	—

TABLE 3
SHEEP MUCOSAL HEPARIN PREPARATION IN TERMS OF THE PORCINE MUCOSAL HEPARIN PREPARATION

Assay method	Lab. no.	No. of assays	Mean potency ratio	Confidence limits (P = 0.95)
British Pharmacopoeia (1963)	3	6	0.492	0.469–0.517
	4	6	0.486	0.474–0.498
	5	6	0.476	0.466–0.487
	8	7	0.522	0.512–0.562
	9	11	0.582	0.575–0.590
	11	6	0.466	0.451–0.482
	12	3	0.510	0.477–0.545
United States Pharmacopeia (1963)	1	18	0.529	0.518–0.541
	2	16	0.494	0.478–0.510
	4	5	0.481	0.469–0.493
	5	16	0.522	0.504–0.542
	7	12	0.481	0.474–0.488
	11	10	0.452	0.439–0.466
	13	12	0.540	0.531–0.549
Clot protein	3	13	0.482	0.476–0.487
Thrombin titration	6	8	0.538	0.529–0.546
Howell	10	38	0.526	0.493–0.561
Colorimetric—DK2	10	2	0.452	—
Colorimetric—Lovibond	10	2	0.454	—

boplastin time and lipoprotein lipase were made for each dose level of each preparation. However, for any of the different response measurements, there was only 1 response to each of the dose levels and so it was not possible to carry out analysis of variance for these assays. The log dose–response lines of each of 3 measurements in turn were examined by eye and any preparation which had a response line with either no slope at all, or a reversed one, was omitted from computation for that assay. Relative potencies of as many as possible of the 7 different comparisons between preparations were calculated 3 times, for each assay, by relating each of the response measurements in turn, to the log dose. These potencies are given in Table 9 together with an unweighted mean potency and confidence limits, based on the direct estimate of variance of the log potencies, for each set of potencies for each type of response.

The estimates of potency using the 3 types of response for any one comparison of 2 preparations in an assay are not truly independent; hence, it was possible to include only 1 of each of the potencies when estimates from these *in vivo* assays were compared or combined with those from the *in vitro* assays in this study. As there were more estimates of potency derived from the measurements of clotting time, these potencies have been chosen to represent the *in vivo* assays from Laboratory 10. It can be seen from Table 9 that all 3 response variables give similar mean potencies.

No consistent difference between the slopes of the log dose–response lines was found when the slopes of the lines of all the preparations were compared within each of the assays using the *in vitro* and *in vivo* assay methods mentioned so far. Analysis of variance of the BP, clot-protein and thrombin-titration assays showed that in several instances the

TABLE 4

PORCINE MUCOSAL HEPARIN PREPARATION IN TERMS OF THE INTERNATIONAL STANDARD FOR HEPARIN ESTABLISHED IN 1958

Assay method	Lab. no.	No. of assays	Mean potency (IU/ampoule)	Confidence limits (P = 0.95: IU/ampoule)
British Pharmacopoeia (1963)	3	9	1 345	1 274–1 419
	4	8	1 438	1 401–1 476
	5	6	1 277	1 164–1 402
	8	6	1 336	1 314–1 358
	9	11	1 203	1 152–1 256
	11	6	1 364	1 314–1 417
	12	4	1 376	1 309–1 447
United States Pharmacopeia (1965)	1	18	1 242	1 221–1 264
	2	24	1 405	1 275–1 548
	4	5	1 232	1 180–1 285
	5	16	1 360	1 302–1 421
	7	12	1 160	1 153–1 167
	11	10	1 194	1 156–1 234
	13	13	1 245	1 221–1 272
Clot protein	3	13	1 374	1 304–1 449
Thrombin titration	6	8	1 323	1 288–1 359
Howell	10	44	1 665	1 580–1 753
Colorimetric—DK2	10	2	1 005	—
Colorimetric—Lovibond	10	2	905	—
In vivo (clotting time)	10	11	1 379	1 182–1 609

slopes of the log dose–response lines were significantly different at the 1% level.

It became apparent in this study that in a large number of the assays analysed, there was little or no variation in the responses (usually clotting times) to a given dose level for any one preparation. No statistical inference should be attached to an analysis of variance carried out on data where it is believed that the responses are not normally distributed within each set. In view of this and the fact that the slopes of the log dose–response lines for some preparations were not consistently greater (or less) than those for other preparations, it was decided to examine the distribution of each of the 7 sets of log potencies from assays where the same assay method used for each laboratory, first including and then excluding the estimates that came from assays where there were significant deviations ($P<0.01$) from parallelism. This showed that the inclusions of the estimates from " non-parallel " assays made no significant difference to the mean and variance of most of the distributions and so all estimates were included in further calculations.

The Howell assays carried out by Laboratory 10, like the *in vivo* assays submitted by the same Laboratory, consisted of 1 response for each dose level. No numerical values were given to the responses; a symbolic notation was used, however, to indicate the degree of clotting as judged visually. In order to calculate potencies, it was necessary to assign numerical values to the degrees of clotting recorded. An assay was rejected as invalid and no computations were carried out if the responses to one preparation lay beyond the range of the responses to the other preparation.

The estimates of potency from the USP assays

TABLE 5

PORCINE MUCOSAL HEPARIN PREPARATION IN TERMS OF THE BRITISH STANDARD FOR MUCOSAL HEPARIN

Assay method	Lab. no.	No. of assays	Mean potency (British units/ampoule)	Confidence limits (P = 0.95: British units/ampoule)
British Pharmacopoeia (1963)	3	14	1 394	1 337–1 454
	4	14	1 357	1 323–1 393
	5	6	1 335	1 299–1 372
	8	9	1 178	1 132–1 226
	9	11	1 018	964–1 076
	11	12	1 263	1 245–1 278
	12	7	1 320	1 290–1 352
United States Pharmacopeia (1965)	1	18	1 441	1 368–1 518
	2	16	1 308	1 271–1 346
	4	5	1 243	1 184–1 306
	5	16	1 370	1 324–1 417
	7	12	1 297	1 290–1 303
	11	10	1 287	1 217–1 361
	13	24	1 331	1 322–1 339
Clot protein	3	13	1 484	1 466–1 502
Thrombin titration	6	8	1 735	1 682–1 791
Howell	10	36	1 935	1 816–2 061
Colorimetric—DK2	10	2	1 104	—
Colorimetric—Lovibond	10	2	1 085	—
In vivo (clotting time)	10	8	1 577	1 401–1 776

TABLE 6

PORCINE MUCOSAL HEPARIN PREPARATION IN TERMS OF THE USP REFERENCE STANDARD FOR SODIUM HEPARIN

Assay method	Lab. no.	No. of assays	Mean potency (USP units/ampoule)	Confidence limits (P = 0.95: USP units/ampoule)
British Pharmacopoeia (1963)	3	2	1 192	—
	4	6	1 115	1 069–1 163
	5	6	1 201	1 146–1 260
	8	7	1 121	1 109–1 133
	9	9	1 071	1 022–1 122
	11	6	1 175	1 131–1 221
	12	3	1 187	1 078–1 308
United States Pharmacopeia (1965)	1	16	1 191	1 170–1 213
	2	21	1 290	1 264–1 317
	4	5	1 280	1 210–1 365
	5	6	1 107	1 050–1 166
	7	9	1 161	1 151–1 170
	11	10	1 355	1 319–1 392
	13	10	1 153	1 124–1 183
Clot protein	3	11	1 259	1 212–1 308
Thrombin titration	6	8	1 156	1 081–1 235
Howell	10	26	1 027	945–1 115
Colorimetric—DK2	10	2	1 298	—
Colorimetric—Lovibond	10	2	1 106	—

TABLE 7

THE BRITISH STANDARD FOR MUCOSAL HEPARIN IN TERMS OF THE INTERNATIONAL STANDARD FOR HEPARIN ESTABLISHED IN 1958

Assay method	Lab. no.	No. of assays	Mean potency (IU/ampoule)	Confidence limits (P = 0.95: IU/ampoule)
British Pharmacopoeia (1963)	3	6	1 186	1 037–1 358
	4	4	1 313	1 218–1 416
	9	11	1 477	1 383–1 577
	11	6	1 360	1 301–1 422
	12	2	1 327	—
United States Pharmacopeia (1965)	1	15	1 093	1 033–1 156
	4	5	1 238	1 173–1 308
	7	12	1 117	1 113–1 123
	11	10	1 160	1 082–1 243
	13	13	1 170	1 148–1 196
Clot protein	3	13	1 158	1 092–1 227
In vivo (clotting time)	10	7	1 106	821–1 489

were calculated in accordance with the recommendations given in the United States Pharmacopeia (1965).

The data for the *in vivo* assays carried out by Laboratory 9 consisted of replicate responses to only 1 dose level of each of the preparations compared, thus it was not possible to do any statistical analysis or to estimate any potencies.

The frequency distributions of the estimates of log potency of the porcine mucosal heparin in terms

TABLE 8

USP REFERENCE STANDARD FOR SODIUM HEPARIN IN TERMS OF THE INTERNATIONAL STANDARD FOR HEPARIN ESTABLISHED IN 1958

Assay method	Lab. no.	No. of assays	Mean potency (IU/mg)	Confidence limits (P = 0.95: IU/mg)
British Pharmacopoeia (1963)	3	1	3 321	—
	4	2	3.660	—
	9	9	3.187	3.007–3.378
	11	2	3.329	—
	12	1	3.291	—
United States Pharmacopeia (1965)	1	14	2.928	2.885–2.972
	4	6	2.708	2.592–2.829
	7	9	2.812	2.786–2.837
	11	10	2.469	2.415–2.524
	13	2	3.046	—
Clot protein	3	11	3.050	2.861–3.251

TABLE 9

ESTIMATED POTENCIES FROM *IN VIVO* ASSAYS CARRIED OUT BY LABORATORY 10

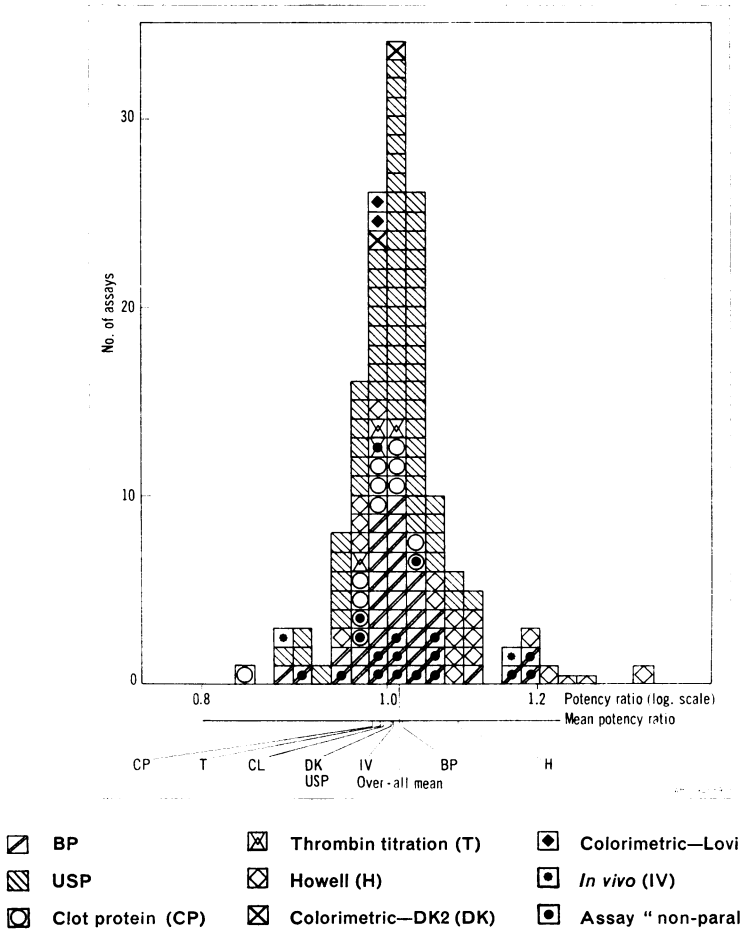
Dog no.	Porcine mucosal heparin and itself (relative potency)			Porcine mucosal heparin preparation and the International Standard (IU/ampoule)			Porcine mucosal heparin preparation and British standard for mucosal heparin (British units/ampoule)			British standard for mucosal heparin and International Standard for Heparin (IU/ampoule) [b]		
	Clotting time	PTT [a]	Lipoprotein lipase	Clotting time	PTT [a]	Lipoprotein lipase	Clotting time	PTT [a]	Lipoprotein lipase	Clotting time	PTT [a]	Lipoprotein lipase
44	—	—	—	2 177	1 306	1 206	1 801	1 664	2 462	1 512	981	981
45	—	—	—	980	1 115	1 606	1 971	1 134	1 279	621	1 570	1 228
46	—	—	—	—	—	—	—	1 397	1 516	—	—	—
47	—	—	—	1 786	—	—	1 622	6 082	2 460	1 376	—	—
48	—	—	—	1 315	—	—	—	—	—	—	—	—
50	—	—	—	—	309	—	1 635	1 718	893	—	273	—
52	—	—	—	1 539	—	—	1 355	1 938	—	1 421	—	—
53	—	—	—	1 008	—	729	1 504	—	2 176	838	—	419
54	—	—	—	1 196	1 532	—	1 273	2 364	1 843	1 174	1 040	—
56	—	—	—	1 378	—	1 121	1 569	—	2 043	1 119	—	686
60	—	—	—	1 431	1 304	—	—	—	—	—	—	—
64	0.880	1.017	0.887	1 374	1 233	1 122	—	—	—	—	—	—
65	1.161	0.742	—	1 365	1 426	1 372	—	—	—	—	—	—
Mean potency	1.011	0.868	—	1 379	1 068	1 160	1 577	1 926	1 800	1 106	765	725
Confidence limits (P = 0.95)	0.173–5.890	0.117–6.436	—	1 182–1 609	639–1 785	878–1 533	1 401–1 776	1 170–3 163	1 321–2 453	821–1 489	253–2 305	299–1 757

[a] PTT = Partial thromboplastin time.

[b] The data for the British and International Standards are the same as used when computing the porcine standard in terms of these 2 standards.

FIG. 1

FREQUENCY DISTRIBUTION OF THE LOG POTENCY RATIOS OF THE PORCINE MUCOSAL HEPARIN PREPARATION IN TERMS OF ITSELF



of itself and in terms of each of the 3 standards, and the log potencies estimated for the British standard, the USP Reference Standard and the sheep mucosal heparin are given in Fig. 1–7. The distribution of potencies for each assay method has been indicated, as have the potencies from " non-parallel " assays, but information on the original source (i.e. laboratory) has not been included in the figures.

In this collaborative study there were many assays whose design led to the use of analysis of variance. However, since (as already mentioned) the distributions of responses was such that a reliable estimate of the error variance could not be made, weights (reciprocal of the variance of the log potency) could not be computed. The design of the remaining assays precluded the calculation of weights. Thus, in this study, it has not been possible to carry out homogeneity tests, as is the usual practice in collaborative studies (Bangham & Woodward, 1966). Unweighted geometric mean potencies and confidence limits, based on the direct estimate of variance of the log potencies, have been computed for each of the 7 comparisons from each of the assay methods used by each laboratory. These results are given in Tables 2–8.

The potencies submitted by Laboratory 10 for their colorimetric assays have been included in the

FIG. 2
FREQUENCY DISTRIBUTION OF THE LOG POTENCIES OF THE PORCINE MUCOSAL HEPARIN PREPARATION IN TERMS OF THE INTERNATIONAL STANDARD FOR HEPARIN ESTABLISHED IN 1958

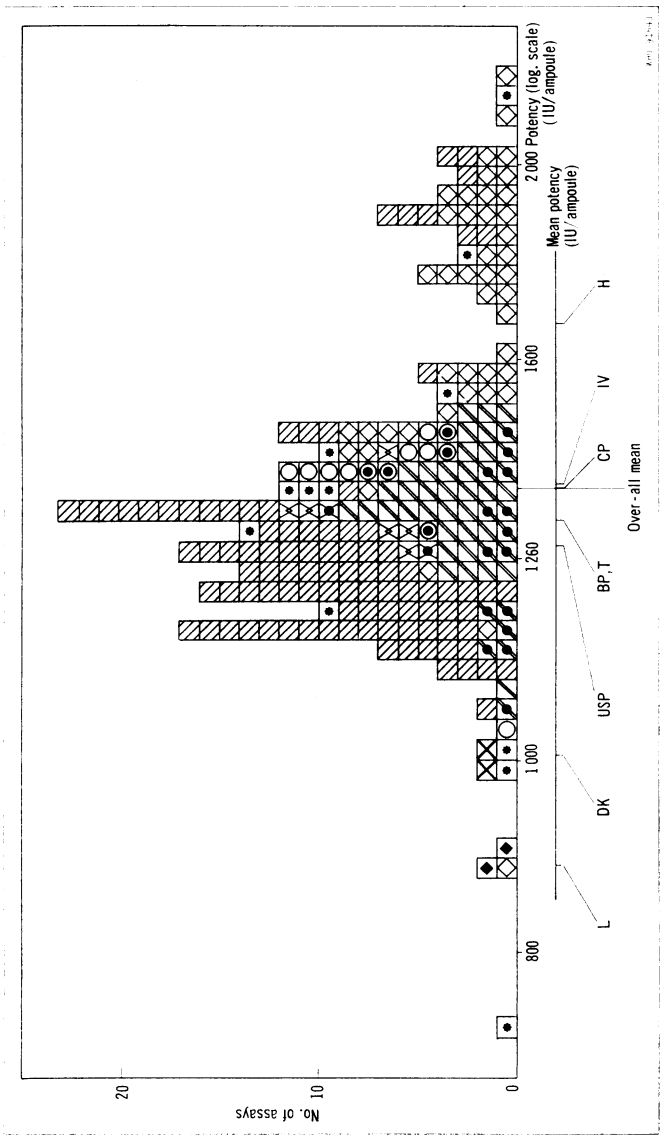


FIG. 3

FREQUENCY DISTRIBUTION OF THE LOG POTENCIES OF THE PORCINE MUCOSAL HEPARIN PREPARATION IN TERMS OF THE BRITISH STANDARD FOR MUCOSAL HEPARIN

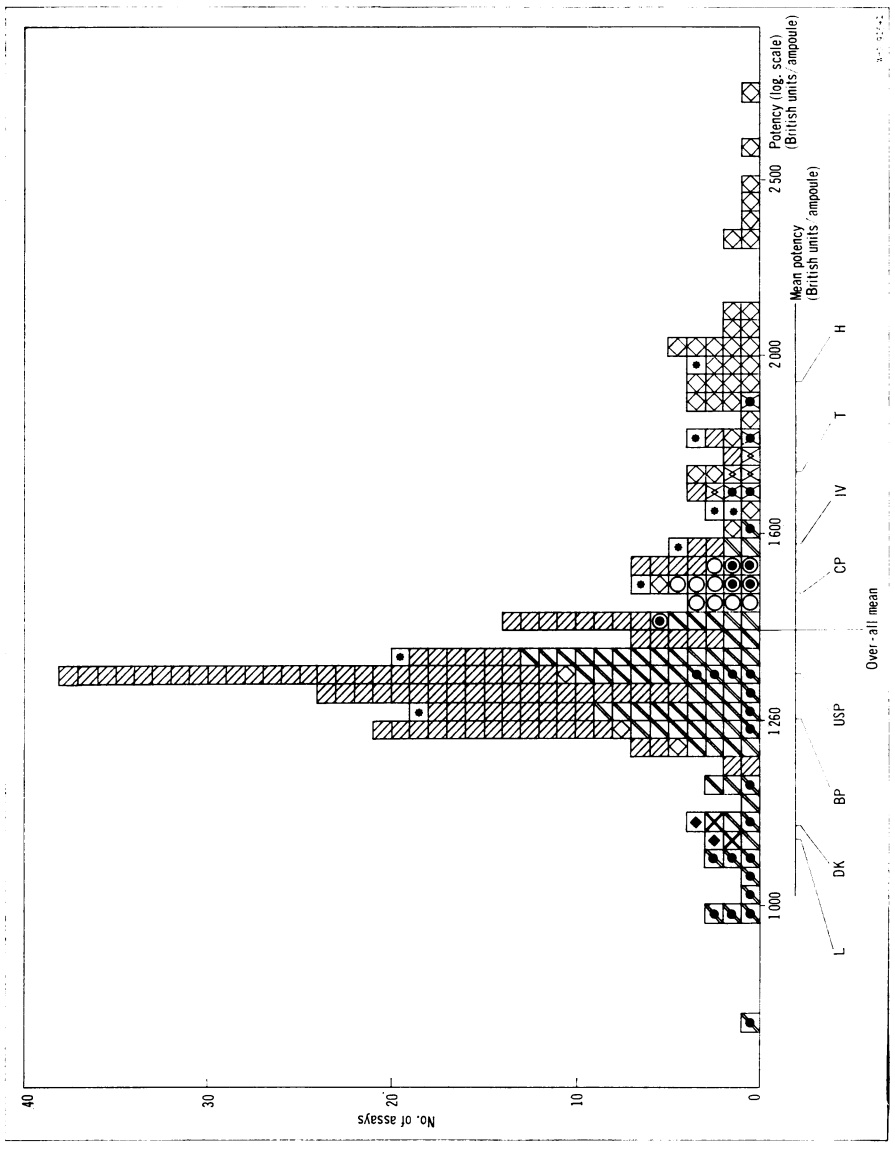


FIG. 4

FREQUENCY DISTRIBUTION OF THE LOG POTENCIES OF THE PORCINE MUCOSAL HEPARIN PREPARATION IN TERMS OF THE USP REFERENCE STANDARD FOR SODIUM HEPARIN

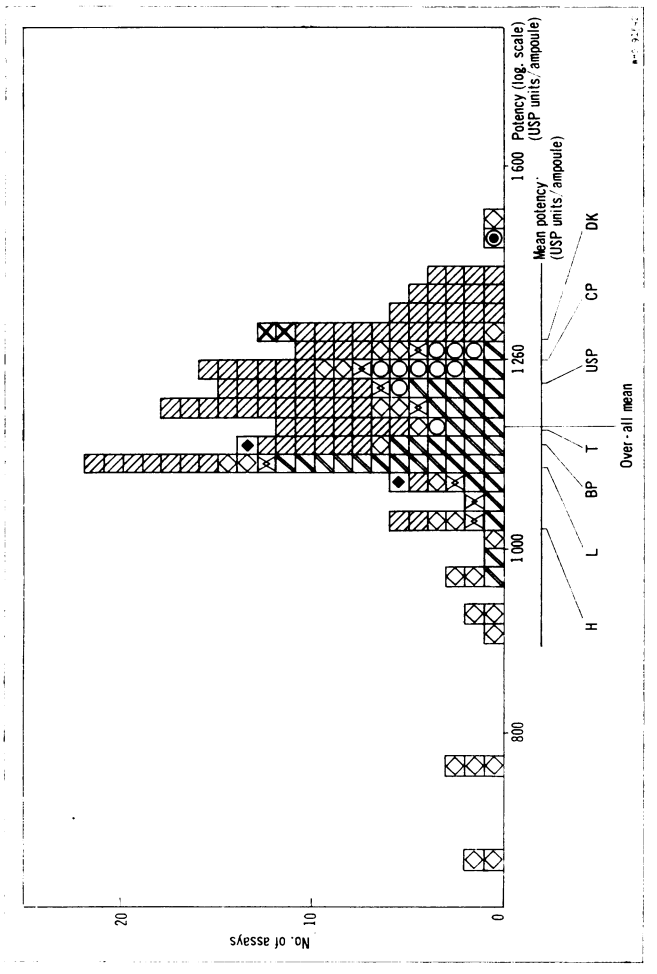


FIG. 5
FREQUENCY DISTRIBUTION OF THE LOG POTENCIES OF THE SHEEP MUCOSAL HEPARIN PREPARATION IN TERMS OF THE PORCINE MUCOSAL HEPARIN PREPARATION

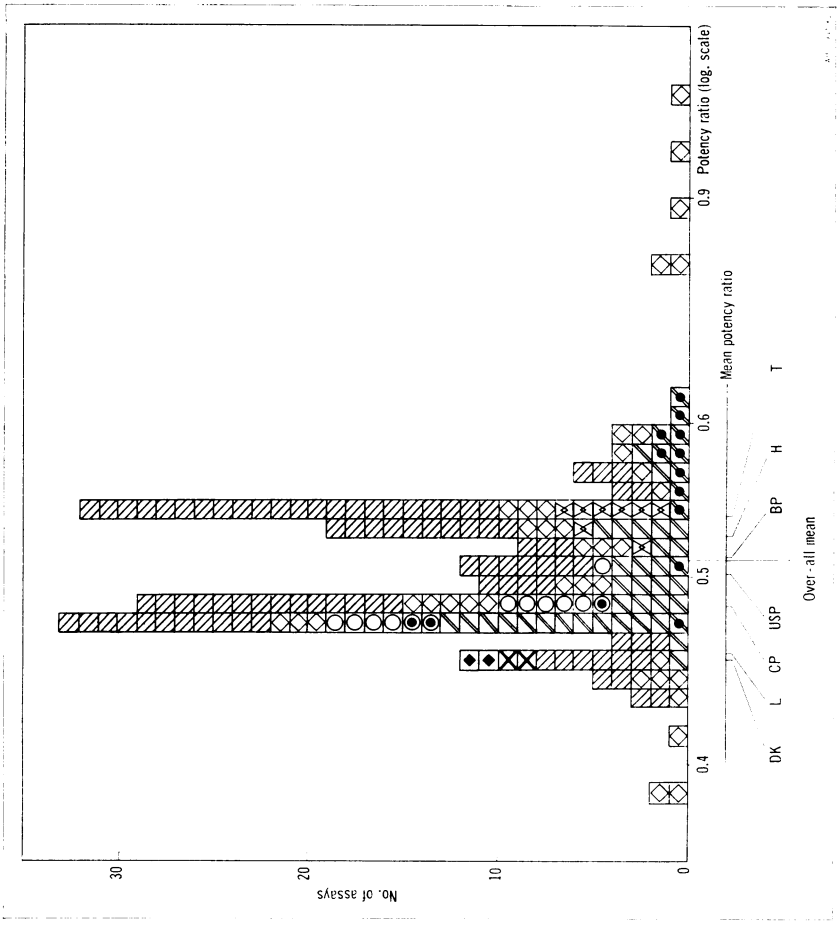


FIG. 6

FREQUENCY DISTRIBUTION OF THE LOG POTENCIES OF THE BRITISH STANDARD FOR MUCOSAL HEPARIN IN TERMS OF THE INTERNATIONAL STANDARD FOR HEPARIN ESTABLISHED IN 1958

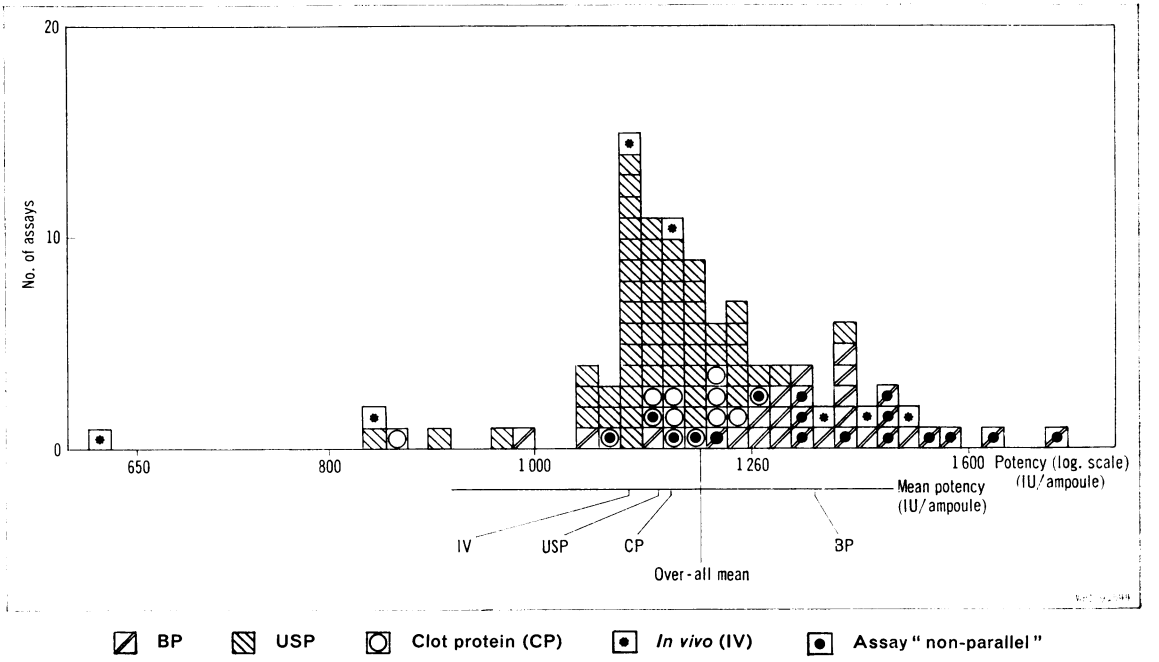


FIG. 7

FREQUENCY DISTRIBUTION OF LOG POTENCIES OF THE USP REFERENCE STANDARD FOR SODIUM HEPARIN IN TERMS OF THE INTERNATIONAL STANDARD FOR HEPARIN ESTABLISHED IN 1958

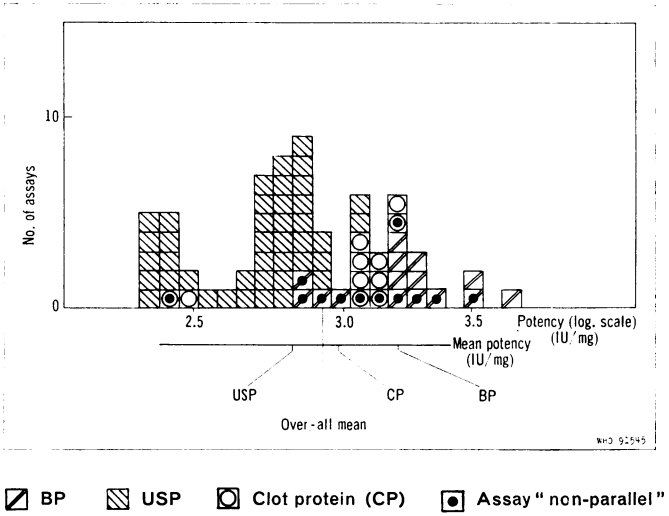


TABLE 10

MEAN POTENCIES AND CONFIDENCE LIMITS FOR EACH ASSAY METHOD

Assay method	No. of assays	Mean potency	Confidence limits (P = 0.95)	Range
Porcine mucosal heparin preparation *versus* itself				
		Potency ratio		
BP	39	1.016	0.996–1.035	0.873–1.179
USP	67	1.006	0.995–1.017	0.894–1.079
Clot protein	13	0.982	0.951–1.013	0.841–1.033
Thrombin titration	4	0.991	0.971–1.012	0.976–1.006
Howell	18	1.086	1.033–1.142	0.942–1.371
Colorimetric—DK2	2	1.007	—	0.995–1.021
Colorimetric—Lovibond	2	0.994	—	0.993–0.995
In vivo (clotting time)	2	1.011	—	0.880–1.161
Sheep mucosal heparin preparation *versus* porcine mucosal heparin preparation				
		Potency ratio		
BP	45	0.512	0.499–0.526	0.445–0.620
USP	89	0.504	0.433–0.588	0.428–0.573
Clot protein	13	0.482	0.476–0.487	0.468–0.506
Thrombin titration	8	0.538	0.529–0.546	0.518–0.549
Howell	38	0.526	0.493–0.561	0.382–0.898
Colorimetric—DK2	2	0.452	—	0.445–0.456
Colorimetric—Lovibond	2	0.454	—	0.451–0.456
Porcine mucosal heparin preparation *versus* International Standard				
		Potency (IU/ampoule)		
BP	50	1 322	1 292–1 352	1 071–1 504
USP	98	1 283	1 248–1 319	1 063–2 011
Clot protein	13	1 374	1 304–1 449	1 040–1 456
Kjems & Wagner	8	1 323	1 288–1 359	1 281–1 415
Howell	44	1 665	1 580–1 753	888–2 195
Colorimetric—DK2	2	1 005	—	994–1 015
Colorimetric—Lovibond	2	905	—	887– 905
In vivo (clotting time)	11	1 379	1 182–1 609	979–2 177
Porcine mucosal heparin preparation *versus* British standard for mucosal heparin				
		Potency (British units/ampoule)		
BP	73	1 264	1 229–1 299	851–1 600
USP	101	1 339	1 319–1 359	1 179–1 810
Clot protein	13	1 484	1 446–1 502	1 424–1 520
Thrombin titration	8	1 735	1 682–1 791	1 662–1 863
Howell	36	1 935	1 816–2 061	1 206–2 868
Colorimetric—DK2	2	1 104	—	1 094–1 115
Colorimetric—Lovibond	2	1 085	—	1 072–1 099
In vivo (clotting time)	8	1 577	1 401–1 776	1 273–1 971

TABLE 10 *(concluded)*

Assay method	No. of assays	Mean potency	Confidence limits (P = 0.95)	Range
Porcine mucosal heparin preparation *versus* USP reference standard for sodium heparin				
		Potency (USP units/ ampoule)		
BP	39	1 137	1 115–1 159	966–1 283
USP	77	1 228	1 207–1 248	1 047–1 411
Clot protein	11	1 259	1 212–1 308	1 150–1 462
Thrombin titration	8	1 156	1 081–1 235	1 047–1 284
Howell	26	1 027	945–1 115	684–1 483
Colorimetric—DK2	2	1 298	—	1 292–1 307
Colorimetric—Lovibond	2	1 106	—	1 089–1 127
British standard for mucosal heparin *versus* International Standard				
		Potency (IU/ampoule)		
BP	29	1 355	1 296–1 417	995–1 757
USP	55	1 142	1 118–1 166	847–1 383
Clot protein	13	1 158	1 092–1 227	867–1 278
In vivo (clotting time)	7	1 106	821–1 489	621–1 511
USP reference standard for sodium heparin *versus* International Standard				
		Potency (IU/mg)		
BP	15	3.281	3.148–3.426	2.914–3.720
USP	41	2.757	2.693–2.823	2.406–3.139
Clot protein	11	3.050	2.861–3.251	2.510–3.260

figures and in Tables 2–8 but excluded from any further calculations.

The estimates of log potency of the porcine mucosal heparin in terms of itself have been combined over all laboratories to give a mean potency and confidence limits for each assay method. These results together with the range of potencies for each assay method are given in Table 10. These computations were also carried out for the other comparisons and the corresponding results are given in the same table (Table 10).

The over-all unweighted geometric mean potencies, confidence limits and range of potencies for each comparison are given in Table 11.

DISCUSSION

This collaborative study was planned to study the problems of comparing (1) different preparations of heparin by (2) different bioassay methods; examination of the results, however, immediately drew attention to the statistical problem, (3) the selection of a suitable method to test the results for statistical validity. Since the last is germane to the evaluation of the first two, the problems are discussed in reverse order.

Tests for statistical validity

In this study the BP, clot-protein and thrombin-titration assays analysed by the standard method for parallel line assays were all found to have a significant ($P<0.05$) regression of the log dose–response lines. When using this form of statistical analysis it is customary to reject as statistically invalid, assays where the slopes of the log dose–response lines are significantly different at the 1% level (Bangham & Woodward, 1966). The test for non-parallelism compares the mean sum of squares for differences between the slopes with the mean sum of squares for the residual error. In these assays there was

TABLE 11
OVER-ALL MEAN POTENCIES AND CONFIDENCE LIMITS

Heparin preparations compared	No. of assays	Mean potency	Confidence limits (P = 0.05)	Range
		Potency ratio		
Porcine mucosal heparin, porcine mucosal heparin	143	1.016	1.005–1.027	0.841–1.371
Sheep mucosal heparin, porcine mucosal heparin	193	0.510	0.502–0.518	0.382–0.898
		Potency (units/ampoule)		
Porcine mucosal heparin, International Standard	224	1 371 IU	1 342–1 401	887–2 195
Porcine mucosal heparin, British standard	239	1 418 British units	1 385–1 452	851–2 868
Porcine mucosal heparin, USP reference standard	161	1 169 USP units	1 150–1 189	684–1 483
British standard, International Standard	104	1 197 IU	1 164–1 231	621–1 757
USP reference standard, International Standard	67	2.915 IU/mg	2.840–2.992	2.406–3.720

often little or no variation in the responses to any one dose level, resulting in the error variances being very small, so that even a slight difference in the slopes of the lines would appear to be significant. Since the analysis of variance carried out on such data is meaningless for testing the statistical validity of assays, other tests had to be applied. These consisted of examining the log dose–response line for each preparation in every assay and if all the lines had a slope of the same sign it was concluded that there was a regression. The slopes of the lines in each assay were ranked and the correlation of slope ranking with preparations was assessed over all assays. This test, used in place of the conventional " deviations-from-parallelism " test, failed to show that one preparation tended to have a steeper or flatter slope than any of the remaining preparations. In addition, examination of the distributions of log potencies with and without the estimates from " non-parallel " assays showed that the inclusion of the potencies did not alter the distributions significantly. It can be seen from the figures that very few of the log potencies from " non-parallel " assays lie on the extremes of the distributions. In most cases where they do, they all come from one laboratory, and the remaining assays from that laboratory also lie in the same part of the distribution. In this collaborative study it has been decided to include all estimates of potency from the BP, clot-protein and thrombin-titration assays.

As reliable error variances could not be estimated, weights could not be attached to the estimated log potencies or the homogeneity of log potencies evaluated, and only unweighted geometric mean potencies and confidence limits could be calculated.

The implication of this is that determination of a " best estimate " of potency of a heparin preparation requires the combination of results from *several completely independent assays*; that is to say, assays using different batches of substrate and activator (when one is used) as well as fresh dilutions of test and standard heparins.

Assay methods

In Tables 10 and 11 the range of individual estimated potencies has been given in addition to the calculated confidence limits of the means, because it is necessary to appreciate the wide scatter of potencies that has been obtained within an assay method for most comparisons of different preparations; this can also be observed in Fig. 1–7. The over-all range in nearly every case was of the order of plus and minus half the mean potency.

Table 2 shows the order of precision obtained with the methods used when one coded preparation was assayed against itself, but all comparisons of different preparations gave divergent estimates. The estimates of potency from the Howell assays cover the widest range of values; this may be partially accounted for however by the fact that the estimates were derived from discontinuous (i.e., non-numerical descriptive) data and also that each assay represents an assay on a different substrate, since blood from a different dog was used for each assay. There is

at present no general agreement as to which method of assay most closely reflects the true biological potency of heparin.

Pharmacopoeial bioassay methods for heparin were devised several years ago, and all have been criticized in the light of modern knowledge of the clotting mechanism. Thus, thrombokinase, like thrombin, is believed to activate only at the later stages in the " enzyme cascade " of the coagulation process, whereas heparin may also act at earlier stages. Sulfated whole ox blood (the substrate for the BP method) tends to be unpredictably unsatisfactory unless taken by venipuncture. The endpoint of the USP method relies on comparative assessment which is somewhat subjective; the " clot protein assay " is an attempt to make it less subjective. *In vivo* assays require scrupulous technique to maintain the animal in a good physiological state, and are impracticable as routine assays; moreover the *ex vivo* blood samples require a more reproducible method of activation than the contact normally obtained with syringes and test tubes. However, whereas *in vitro* assays are influenced by heparin molecules of all sizes, *in vivo* assays presumably reflect the anticoagulant activity due only to those heparin molecules that are large enough not to be rapidly excreted by the kidney.

Although coming from a wide range of individual estimates, the mean potencies for the *in vivo* assays (when clotting time was the response measured) agree well with the results from the majority of the *in vitro* assays. Differences in estimates of potency can be partially explained by the fact that only 1 dog was used for each assay; it has been shown in other work by Laboratory 10 (personal communication) that there is usually a significant difference between dogs, so it is to be expected that any estimate of potency based on 1 dog would have very wide confidence limits. This additional work also showed that when various heparins were assayed against one another and the design of the assay was such that the analysis for parallel line assays could be carried out, the assays were statistically valid in the sense that the regression of the log dose–response line was significant and that the deviations from parallelism were not significant. This justified the crude tests for validity that were carried out for the assays in this study.

The estimated potencies from the colorimetric assays agree with the estimates obtained from biological assays when the porcine mucosal heparin was compared with itself and the USP reference standard, but in other sets of comparisons the estimates tended to be lower than the biological assay results. There is thus no indication that colorimetric assays can be relied upon to reflect biological potency.

Differences between heparin preparations

This study was instigated partly as a result of observations on the heterogeneity of heparin preparations from oxen, sheep and pigs (although recent reports have shown that heparin obtained from whales differs markedly in certain respects from the preparations used in this study, a sample of it was not included). As explained in the introduction, the relative potencies of some preparations made from intestinal mucosa, when assayed against the International Standard, were found to be consistently higher by the BP method than by the USP method. This tendency was confirmed in the assays of the ox mucosal heparin (British standard) when the confidence limits for the combined assays do not overlap (Table 10, and Fig. 6) and to a lesser extent with the other mucosal preparations (Tables 10 and 11 and Fig. 4 and 7).

The difference in the amount of protamine required to neutralize the anticoagulant activity of different preparations was not studied. Unfortunately the importance of this did not come to light until after this study had been planned and started. Several participants have since commented on the desirability of this aspect being investigated as there is evidence that more than 1 mg of protamine is required to neutralize 1 mg of the preparations with high specific activity (150 IU/mg and above).

Possible need for replacement of the International Standard

Of the several respects in which preparations of heparin may vary, those due to species and tissue of origin included in this study do not appear to give differences in relative potencies (by two widely used pharmacopoeial methods) that are consistently so large as to necessitate another separate international standard.

The Expert Committee on Biological Standardization (1969) reported the following:

> The Committee noted ... the results of the collaborative assay, referred to in its nineteenth report, ... of preparations of heparin, extracted from lungs and from intestinal mucosa of sheep, swine and cattle. The results showed that with two commonly used procedures for assaying heparins, the need referred to in the seventeenth

report ... for a separate international standard for mucosal heparin was not confirmed.

We suggest that the pig mucosal preparation included in this study should in due course replace the current International Standard when stocks are exhausted, because it is more representative of modern preparations than the current standard. The preparation was ampouled in a way which would make it acceptable and it has now been calibrated by some 224 assays using different methods and also compared with 2 national standards. If this preparation is accepted it is suggested that the figure of 1370 IU/ampoule (based on the mean estimate of 1371 IU/ampoule, Table 11, confidence limits 1342–1401, P = 0.95) would be a reasonable figure on which to base the unitage assigned to each ampoule.

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RÉSUMÉ

ÉTUDE COLLECTIVE D'HÉPARINES D'ORIGINES DIFFÉRENTES

Jusqu'il y a une dizaine d'années, l'héparine était préparée à partir de tissu pulmonaire de bœuf; son activité biologique, relativement constante, était de l'ordre de 100 UI/mg. Actuellement, la muqueuse intestinale du porc, du mouton et des bovins représente la principale source d'approvisionnement pour la fabrication industrielle du produit. Ces préparations d'héparine d'origine muqueuse, mélanges de composants aux caractéristiques chimiques et physiques souvent différentes, font preuve d'une activité biologique beaucoup plus spécifique. On a signalé à plusieurs occasions une divergence des résultats obtenus lors des essais biologiques effectués avec ces deux types d'héparine par les méthodes courantes; s'il en est bien ainsi, on peut redouter que ces discordances entraînent une confusion aussi bien dans le domaine commercial qu'en ce qui regarde le contrôle par les organismes nationaux compétents et l'utilisation clinique du produit.

Une étude internationale a été organisée avec pour objectif de jeter quelque lumière sur cette question et sur des problèmes connexes. On a procédé dans 13 laboratoires de 7 pays à un titrage comparatif portant sur 5 préparations d'héparine: l'étalon international (à base de tissu pulmonaire de bœuf) établi en 1958; deux préparations nationales de référence; une héparine de muqueuse intestinale de porc; une héparine de muqueuse intestinale de mouton. Neuf méthodes différentes ont été utilisées pour ces essais.

Les résultats ont fait apparaître des différences d'activité relative entre héparine préparée à partir de tissu pulmonaire et héparines de muqueuse intestinale. Ces différences n'étaient cependant pas observées avec une régularité justifiant la création de deux étalons internationaux distincts, bien que l'imprécision des essais ait pu masquer une hétérogénéité fondamentale des deux types de préparation. La reproductibilité des résultats était très satisfaisante lorsque les deux méthodes les plus courantes étaient appliquées à une même préparation. Par contre, les essais effectués par différents laboratoires ou portant sur différents lots d'une même préparation ont fourni des données très variables.

Selon les auteurs, l'activité biologique des préparations d'héparine devrait être évaluée au cours d'essais indépendants effectués sur des lots différents. Les méthodes présentement en usage, qui datent de 15 à 20 ans, s'avèrent peu satisfaisantes à maints égards. On suggère de remplacer l'étalon international actuel, dès que le stock en sera épuisé, par un nouvel étalon d'héparine de muqueuse intestinale de porc, mieux adapté au titrage des préparations en usage aujourd'hui.

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