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STUDIES IN OSTEOGENESIS

BY

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Levander showed that when cell-free alcoholic extracts of bone are injected into rabbits' muscles, cartilage and bone form at the site of the injection. This finding has since been confirmed by *Annersten, Bertelsen,* and *Lacroix.* As extracting agents, *Levander* used both ordinary and acidified alcohol. *Annersten,* who extracted mainly with acidified alcohol, was able to show that the active factor could be transferred from alcohol to ether, benzene and oil. He used mostly homozoic material (rabbit), but also obtained positive results in some series with heterozoic material (calf-rabbit). *Bertelsen* tried extracts of each of the various layers of bone—periosteum, hard bone, epiphyses and marrow—and found osteogenic activity in all: the extracts from bone marrow gave the best results. *Annersten* believed that the active factor belonged to the lipoid or sterol groups, in view of its solubility in benzene and oil. *Bertelsen* disagreed. Finally, *Blum* observed bone formation in rabbit muscle "in a few cases" after the injection of extracts prepared by Robinson's method for the preparation of phosphatase.

Levander found no cartilage or bone formation in 80 control tests in which he had used injections of pure alcohol and of extracts of connective tissue or muscle. *Annersten* found cartilage formation in 4 out of 102 control tests. Statistical treatment of his material shows, however, that "die mit Knochenextrakt erhaltenen positiven Resultate mussen einem in diesem extrakt vorhandenen Faktor zugeschrieben werden,

der in den Kontroll-experimente fehlt". *Bertelsen* found 1 positive result in 41 control tests. *Blum*'s control tests were all negative.

OUR OWN TESTS

Our first tests confirmed *Annersten*'s observation that the active principle in homozoic extracts can be transferred from alcohol to benzene and ether. As it was essential, from the point of view of obtaining material, to use heterozoic material, we injected extracts of calf bone marrow into rabbit's muscles. These heterozoic injections proved as effective as the homozoic. We therefore preferred to work with heterozoic material (calf-rabbit). Since it seemed that it was possible to tranfer the active factor between a number of soluble agents, we made an investigation to discover whether the activity could be collected quantitatively in a particular fraction.

EXPERIMENTAL METHODS AND ASSESSMENT OF THE RESULTS

We used the original method, injecting the extracts into the rectus femoris muscle of rabbits. The connective tissue membrane, which surrounds the rectus femoris like a capsule, is probably of importance in retaining the injected fluid and in preventing its too rapid diffusion into the neighbouring tissues.

The extract was injected on 4 successive days according to the following schedule:

1st day: 1 ml. extract mixed with 1 ml. physiological saline
2nd day: 1 ml. extract mixed with 1 ml. physiological saline
3rd day: ½ ml. extract mixed with ½ ml. physiological saline
4th day: ½ ml. extract mixed with ½ ml. physiological saline

If the extract for injection was dissolved in oil or water we began with an alcohol injection:

1st day: 2 ml. of 40 % alcohol
2nd day: 1 ml. extract
3rd day: 1 ml. „
4th day: 1 ml. „

The extracts were prepared so that 1 ml. was equivalent to 0.5 gm. of bone marrow.

Usually, each extract was tested on 10 rabbits and was injected into both thighs of each animal. Thus, each series of 10 rabbits contained a maximum of 20 specimens. After the last injection we usually waited between 2 and 3 weeks before taking the specimen. The animal was then killed; the rectus femoris muscle was removed and fixed immediately in formalin, and the usual histological preparations were made, the sections being cut longitudinally. Under "number of specimens" are counted only those which showed the possibility of bone formation i.e. an ossifiable environment in the form of a definite granulation tissue with its abundant vessel and mesenchymal formation. A "positive" specimen is one showing definite cartilage or bone formation. The figures in brackets after the percentage of positive specimens in each series show the number of specimens with definite reactive granulation tissue, and the number with bone or cartilage formation. Since some animals die during the observation period, and others show no mesenchymal reaction in the muscle, the number of specimens in each series may be less than the original 20.

Thus, a result expressed as 50 % (14:7) means that out of the original 20 specimens 14 showed reactive tissue, and of these 7 showed cartilage or bone formation.

I. EXTRACTION WITH ACIDIFIED ALCOHOL

Test 1. Preparation: Calf bone marrow
95 % alcohol
Acidified alcohol (1,000 ml. 95 % alcohol + 50 ml. N/10 HCl)
Benzene

Method: 70 gm. marrow was homogenized in a mortar, and then shaken in a bottle with 210 ml. alcohol for 18 hours. After centrifuging a clear supernatant fluid was obtained. This was decanted and concentrated by evaporation in a water bath to 25 ml., when a clear pale yellow liquid with a residue was obtained. These 25 ml. of alcoholic solution were shaken in a separating funnel with 25 ml. of benzene, and two layers were obtained. The upper, benzene, layer was pale yellow and rather clear; the lower, alcoholic, layer was white and milky. The two layers were separated, and the alcoholic layer was shaken up again with 25 ml. of benzene. The benzene layer was now colourless and rather clear, whereas the alcohol layer was still milky. *The alcohol layer:* the benzene was evaporated in a vacuum: the solution became pale yellow, somewhat opalescent and with some residue. It was diluted up to 140 ml. with 95 % alcohol = *extract A* (rather clear and almost colourless). *The benzene layer:* was reduced to complete dryness in a vacuum and was then dissolved in 10 ml. acid alcohol, giving a yellow solution with some residue. This solution was diluted with 130 ml. 95 % alcohol = *extract AB* (clear and almost colourless).

Result: Extract A. 73 % (11:8) positive.
AB. 38 % (16:6) positive.

As it was found that after two shakings, activity still remained in the alcohol fraction, further tests were made, using the same method but more thorough shaking.

In *Test* 2 the solutions were shaken 3 times with benzene, as in Test 1. The benzene fraction obtained gave 7 % positives (14:1). After shaking the same alcoholic solution with benzene 7 more times, no activity could be demonstrated in the combined benzene fractions (12:0). But the alcoholic fraction which had thus been shaken 10 times with benzene still had some activity = 42 % positives (12:5).

In *Test* 3 the combined benzene fractions showed after 7 shakings with the alcoholic layer 11 % positive (18:2), while the alcohol fraction had 33 % positives (18:6). In *Test* 4 *the* combined benzene fractions were tested after only 3 shakings with the alcohol fraction and gave 26 % positives (19:5).

II. EXTRACTION WITH ACIDIFIED ALCOHOL + PETROLEUM ETHER

When the marrow was extracted with acidified alcohol only, it tended to form into round masses, and one could not be certain that the extraction was complete. Therefore, a

combined alcohol and petroleum ether extraction was used and the marrow dispersed to give a homogeneous mixture.

Test 5. Preparation: Calf bone marrow
95 % alcohol
Acidified alcohol (see test 1)
Petroleum ether
Benzene
Olive oil

Method: 70 gm. marrow was homogenised in a mortar, introduced into a bottle together with 210 ml. acid alcohol + 210 ml. petroleum ether, and shaken for 18 hours. It was then centrifuged for about 20 minutes. The alcohol and the petroleum ether separated, and the undissolved substance sedimented out. The two layers were drawn off separately. The *petroleum ether layer* was evaporated in a vacuum until all the petroleum ether had been driven off. This left a mass, which, after cooling, appeared firm and white. It was dissolved in olive oil = *extract P.*

The *alcohol layer* was evaporated on a water bath in a vacuum to a volume of 35 ml. It was then shaken twice with benzene, and the alcohol layer was diluted with 95 % alcohol = *extract A.*

The *benzene layer:* the two benzene layers obtained by shaking with the alcohol layer were combined and evaporated to complete dryness. The residue was dissolved in 10 ml. acidified alcohol, and then diluted to give *extract B.*

Result: Extract P = 44 % positives (9:4)
A = 28 % — (18:5)
B = 23 % — (13:3)

In a similar test, *Test 6,* the alcohol fraction was tested after it had been shaken three times with benzene. *Result* = 40 % positives (10:4), while the combined benzene fractions were inactive (16:0). In the following tests (*Tests* 7 and 8) the combined benzene fractions obtained after 3 shakings of the alcohol fraction were tested alone, and gave 0 % (8:0) and 8 % positives (12:1) respectively.

Test 9. Preparation: As in Test 5 + pure acetone.

Method: After 24 hours' extraction by shaking, the alcohol and petroleum ether layers were decanted separately. The *alcohol layer* was shaken with petroleum ether 3 times, and each petroleum ether layer was shaken with 95 % alcohol.

The *petroleum ether layer* was shaken with 95 % alcohol and the resulting alcohol and petroleum layers were combined.

The *alcohol fraction* was partially evaporated in a vacuum and shaken three times with benzene. The combined benzene layers were treated as in *Test 5* = *extract B*. The remaining alcohol fraction was precipitated with acetone. The precipitate obtained after 24 hours was dried in a dessicator and became yellowish and of a waxy consisteney. It was dissolved in 95 % alcohol = *extract A*.

The *petroleum ether fraction* was evaporated in a vacuum until all the petroleum ether had disappeared. The residue, a yellow fat, was precipitated with acetone, and gave a white fairly plentiful precipitate, which was dried, ground to powder and dissolved in olive oil = P ac. The acetone was evaporated off the filtrate after the precipitation, and one half of the remaining fat was diluted with olive oil = *extract P*.

Result: Extract A	=	0 %	positives	(14:0)
A ac	=	50 %	—	(14:7)
B	=	72 %	—	(18:13)
P ac	=	0 %	—	(16:0)
P	=	0 %	—	(16:0)

Test 10 was carried out like Test 9, except that the acetone precipitate from the alcohol fraction was dissolved in saline solution. In this series nearly all the preparations showed infection, and no bone formation was obtained (20:0). The alcohol fraction, i.e. the filtrate from the acetone precipitate gave 10 % (20:2), the benzene fraction 32 % (19:6), and the acetone filtrate from the petroleum ether fraction 6 % positives (18:1).

Test 11 was also carried out like Test 9, but in addition one benzene fraction was washed with distilled water. The combined water fractions gave 10 % positives (20:2). The benzene fraction shaken with water was dried with sodium sulphate and then dissolved in 95 % alcohol; it gave no positives (20:0). The part of the benzene fraction which had not been washed with water showed 35 % positives (20:7). The alcohol fraction, i.e. the filtrate left after the acetone precipitation was inactive (20:0), as was also the acetone precipitate from the same fraction, dissolved in saline solution (20:0). The acetone precipitate from the petroleum ether fraction dissolved in alcohol showed weak activity = 6 % positives (18:1).

Test 12 was made in the same way as Test 10. The filtrate obtained after acetone precipitation of the alcohol fraction showed 31 % positives (16:5), while the precipitate dissolved in saline gave 40 % positives (20:8). The acetone precipitate from the petroleum ether fraction was inactive (20:0).

In *Test 13*, carried out in the same way as Test 11, the following

fractions were tested: the combined benzene fractions obtained by shaking the alcohol fraction; the alcohol fraction shaken with the benzene; one alcohol fraction which had been washed with distilled water; and the combined water fractions obtained from the benzene washing. All series were negative (20:0).

CHROMATOGRAPHIC TESTS

In *Test 14*, chromatography was used. The petroleum ether fraction from Test 6 was used. This extract is a firm, white fat at room temperature; it was melted for each test.

Apparatus and preparation: Chromatogram tube, 20 mm. diameter.
Aluminium oxide, according to Brockman.
Petroleum ether.

Method: The extract was dissolved in 25 ml. petroleum ether. The chromatogram, which had no coloured zones, was divided into 3 equal columns, each extracted separately in petroleum ether + some methanol.

Columns 1 and 2 from 2 tests were tested. The petroleum ether was evaporated in a water bath. The remaining fat was dissolved in olive oil. *Result:* Both series were inactive (17:0 and 20:0).

In *Test 15* the dried benzene fraction from Test 11 was used as a raw extract; it unfortunately proved to be inactive. The same technique was used. 3 chromatograms were tried. *Result:* Both series were inactive (10:0 and 10:0).

III. TESTS WITH VARIOUS METHODS OF EXTRACTION

1. Acid alcohol + ethyl ether:

In *Test 16* the alcohol-ether layer obtained after the extraction could not be separated. It was evaporated in a vacuum to 35 ml., and shaken twice with benzene. The resulting benzene solutions were combined and evaporated to complete dryness, and the residue was dissolved in alcohol. *Result:* 30 % positives (20:6).

Test 17 was performed in the same way as Test 16, except that the evaporated alcohol-ether layer was shaken with benzene 7 times instead of only twice. The combined benzene layers were evaporated to complete dryness. The residue was a firm, white fat after cooling; one part of it was dissolved in oil, and one part in alcohol. *Result:* the oil solution gave 0 positives (20:0), and the alcohol solution 25 % positives (20:5).

2. Benzene. (Soxhlet):

Test 18. The marrow was dried with Na_2SO_4. After the Soxhlet extraction the benzene solution was evaporated to complete dryness. Part of the residue was dissolved in oil. *Result:* 25 % positives (12:3).

3. 95 % alcohol + petroleum ether:

Test 19. After the extraction, the two layers were separated. Only the alcohol fraction was used. It was partly evaporated, and shaken 3 times with benzene. The combined benzene layers were evaporated to dryness. The residue was dissolved in alcohol. *Result:* 59 % positives (17:10). The alcohol layer was diluted with 95 % alcohol. *Result:* 47 % positives (15:7).

4. Petroleum ether:

Test 20. When the marrow from Test 4 had been shaken with acid alcohol, and the alcohol layer had been filtered off, the rest of the marrow was this time not discarded, but was shaken with petroleum ether. The petroleum ether layer was then evaporated to dryness, and the residue was dissolved in olive oil. *Result:* 0 positives (19:0).

CHEMICAL INVESTIGATIONS OF THE BONE MARROW EXTRACTS

Lieberman-Burchard's sterol reaction was performed on 10 different extracts. It was negative in 4; their total of positives was 54 % (56:30). In the other 6 extracts it was positive; their total of positives was 26 % (108:28).

Tests for phosphatase were made on the extracts of tests 6, 17 and 19, and were all negative.

The *Biuret reaction* was negative in extracts 1 A and 5 A.

The solubility of the acetone precipitates was as follows:

insoluble in olive oil at room temperature
partly soluble in alcohol at 37° C.
completely soluble in distilled water at room temperature.

The water fractions after washing the benzene in Test 13 gave a positive reaction for unsaturated fatty acids (decoloration of $KMnO_4$).

SUMMARY

Levander and others showed that alcoholic extracts of bone and bone marrow contain a factor with a definite osteogenetic

activity, e.g. injection of the extracts into the quadratus femoris of rabbits resulted in the formation of bone or cartilage in the muscle. In the present investigation we have tried to collect the osteogenetic activity quantitatively in one or other of the dissolving agents by shaking an alcoholic extract of bone marrow with benzene. In this we were unsuccessful; in fact it was found that the activity stubbornly remained in the alcohol fraction. After shaking the alcohol extract with two separate solutions of benzene the alcohol fraction gave 73 % positives (Test 1), and the benzene fraction only 38 %. After shaking the alcohol extract with 10 separate benzene solutions, the alcohol fraction was still active (42 % positives), while the last benzene fraction was completely inactive. In the tests with extracts in acid alcohol the alcohol fraction gave 46 % positive results (41:19) and the benzene fraction 17 % (79:14). In tests with extracts in acid alcohol + petroleum ether, the alcohol fraction gave 30 % (98:29), and the benzene fraction 27 % positives (124:33).

In two tests it was possible to collect a considerable activity in the acetone precipitate obtained from an alcohol fraction (40 % and 50 % positives respectively), the filtrate after the precipitation being considerably less active. The combined precipitates gave 28 % (54:15) and the combined filtrates 10 % (70:7) positives.

The activity of the extracts from the petroleum ether fraction was remarkably low; good result (44 %) was obtained in only one test. All the petroleum ether extracts gave an average of only 4 % positives (154:6). After chromatography the extracts were completely inactive; in one test this was probably due to inactivity of the original benzene extract, in the other, a petroleum ether extract, which usually has a very low activity, was used. Therefore, in both cases the original extract was probably inactive.

Of the two water extracts, one gave a weak positive result (10 %) and one was inactive; the former came from a positive (35 %) and the latter from a negative benzene extract.

The rather large number of series with completely negative results, the extracts being altogether inactive, might be regarded as control tests. If all the positive and all the negative series are collected into 2 groups, one obtains the figures 456:137 (30 %), and 437:0. Statistically, this proves that the positives cannot be due to pure chance.

The experiments have shown that the results obtained with heterozoic material are comparable with those obtained with homozoic.

It would be premature to attempt to base any chemical classification of the osteogenic factor on the results obtained here. Some conclusions may, however, be drawn. It is interesting that it is not possible to extract all the active factor out of an alcohol fraction by means of benzene, and that the particular solvent for sterols and lipoids, petroleum ether, fails to extract the active factor from the alcohol. This supports the opinion that the active factor is not a pure sterol or lipoid, which is also confirmed by the low activity of the pure petroleum ether extracts. Further, most extracts gave negative reactions when tested for sterols. The activity of the acetone precipitates from the alcohol extracts suggests a phosphatide, but this is contradicted by their solubility in distilled water, and their relative insolubity in oil.

RESUME

Levander et d'autres ont montré que les extraits d'alcool d'os et de moelle osseuse contiennent un facteur possédant une activité ostéogénétique définie. Ainsi, il résulte de l'injection de ces extraits dans le quadratus femoris des lapins une formation osseuse ou cartilagineuse dans le muscle. Dans les présentes recherches, nous avons essayé de recueillir la donnée quantitative de l'activité ostéogénétique en mélangeant l'extrait d'alcool de moelle osseuse dans divers dissolvants à du benzène. Nous n'avons eu aucun succès ; en réalité it a été constaté que l'activité reste opiniâtrement dans la fraction

d'alcool. Après avoir mélangé l'extrait d'alcool avec des solutions séparées de benzène, la fraction d'alcool donna 73 % de réactions positives (Test 1) et la fraction de benzène 38 % seulement. Après avoir mélangé l'extrait d'alcool avec 10 solutions séparées de benzène, la fraction d'alcohol était encore active (42 % réactions positives), tandis que la dernière fraction de benzène était complètement inactive. Dans les tests avec extraits dans des acides d'alcool, la fraction d'alcool donna 46 % de résultats positifs et la fraction de benzène 17 %. Dans les tests avec des extraits bruts dans des acides d'alcool + éther de pétrole, la fraction d'alcool donna 30 % (41 : 19), et la fraction de benzène 27 % réactions positives (124 : 33).

Dans deux tests, il a été possible de recueillir une activité considérable de la précipitation d'acétone obtenue d'une fraction d'alcool (40 % et 50 % réactions positives respectivement), la substance filtrée après la précipitation étant beaucoup moins active. Les précipitations combinées ont donné 28 % (54 : 15) et les substances filtrées combinées 10 % (70 : 7) positives.

L'activité des extraits de la fraction de l'éther de pétrole était curieusement basse ; un bon résultat (44 %) a été obtenu dans dans un seul des tests. Tous les extraits d'éther de pétrole ont donné une moyenne n'atteignant que 4 % de réactions positives (154 : 6). Aprés la chromophotographie, les extraits étaient complètement inactifs ; dans un test ceci était probablement dû à l'inactivité de l'extrait primitif de benzène, dans l'autre, on a utilisé un extrait d'éther de pétrole dont l'activité est généralement très basse. C'est probablement pourquoi l'extrait original est resté inactif dans ces deux cas.

Sur deux extraits d'eau, l'un a donné un faible résultat positif (10 %) et l'autre est resté inactif ; le premier provenait d'une réaction positive (35 %), le dernier d'un extrait de benzène négatif.

Le nombre relativement élevé de séries donnant des résultats absolument négatifs, les extraits étant dans l'ensemble

inactifs, doivent être considérées comme des tests de contrôle. Si toutes les séries positives et toutes les séries négatives sont rangée en deux groupes, on obtient les données 456 : 137 (30 %) et 437 : 0. D'un point de vue statistique, ceci prouve que les données positives n'ont pas été obtenues par le effet du hasard.

Les expériences ont montré que les résultats obtenus avec un matériel hétérozoique sont comparables à ceux d'un matériel homozoique.

Il serait prématuré de vouloir établir une classification chimique du facteur ostéogénique sur les résultats obtenus ici. On peut toutefois en tirer certaines conclusions. Il est intéressant de constater qu'il n'est pas possible d'extraire tout le facteur actif d'une fraction d'alcool au moyen du benzène, et notamment que le solvant particulier des stérols et des lipoides, l'éther de pétrole, n'arrive pas non plus à extraire le facteur actif de l'alcool. Ceci appuie l'hypothèse que le facteur actif n'est pas un pur stérol ou lipoide, ce qui est confirmé également par la faible activité des extraits de pur éther de pétrole. Par ailleurs, les extraits les plus actifs ont donné des réactions négatives lorsqu'ils ont été essayés comme stérols. L'activité des précipitations d'acétone des extraits d'alcool semble indiquer qu'il y a phosphatisation, mais ceci est contredit par la solubilité des précipitations dans l'eau distillée et leur insolubilité relative dans l'huile.

ZUSAMMENFASSUNG

Levander und andere Authoren zeigten, dass alkoholische Extrakte von Knochen und Knochen-marken einen Faktor mit definitiven osteogentischen Eigenschaften enthalten. Injektion der Extrakte in den quadratus femoris von Kaninen resultierte in der Bildung von Knorpel oder Knochen im Muskel. In den vorliegenden Untersuchungen haben wir versucht die osteogenetische Aktivitet quantitativ in dem einen oder dem anderen der lösenden Agentia zu sammeln, indem wir einen alko-

holischen Extrakt von Knochenmark mit Benzen ausschüttelten. Wir waren damit nicht erfolgreich. Wir fanden in der Tat dass die Aktivität ständig in der Alkoholfraktion zurückblieb. Nach Ausschüttelung des Alkoholextraktes mit zwei gesondeten Benzenlösungen, gab die Alkoholfraktion 73 % positive Resultater (Versuch 1), und die Benzenfraktion nur 38 %. Nach Ausschüttelung des Alkoholextraktes mit 10 gesondeten Benzenlösungen, war die Alkoholfraktion noch immer aktiv (42 % positive), während die letzte Benzenfraktion vollständig inaktiv war. Die Versuche mit Extrakten mit Säure-Alkohol gaben in der Alkoholfraktion 46 % positive Resultater (41:19) und der Benzenfraktion 17 % (79:14). In Versuchen mit Extrakten mit Säure-Alkohol und Petroleumäther gab die Alkoholfraktion 30 % (98:29) und die Benzenfraktion 27 % positive Resultater (124:33).

In zwei Versuchen was es möglich eine bedeutende Aktivitet in dem Azeton Präzipitat, das von der Alkoholfraktion erhalten wurde, zu sammeln (40 % respektive 50 % positive Resultater). Das Filtrat nach der Prebipitation war bedeutend weniger aktiv. Die Präzipitate zusammen gaben 28 % (54:15) und die Filtrate zusammen gaben 10 % (70:7) positive Resultater.

Die Aktivitet der Extrakte der Petroleumätherfraktion war auffallend niedrig. Nur in einem Versuch (44 %) wurde ein gutes Resultat erzielt. Die Petroleumätherextrakte zusammen gaben einen Durchschnitt von nur 4 % positiven Resultaten (156:6). Nach Chromatographie waren die Extrakte vollständig inaktiv. In einem Versuch war die Ursache wahrscheinlich Inaktivität des ursprünglichen Benzenextraktes, in den anderen wurde ein Petroleumätherextrakt, der gewöhnlich eine sehr geringe aktivitet hat, verwendet. Deshalb war in beiden Fällen der ursprüngliche Extrakt wahrscheinlich inaktiv.

Von den zwei wässrigen Extrakten gab einer schwach positive Resultater (10 %) und der andere war inaktiv. Der erstere kam von einem positiven Benzenextrakten (35 %) und der letztere von einem negativen Benzenextrakt.

Die ziehmlich grosse Anzahl von Reihenfolgen mit vollständig negativen Resultat, bei inaktiven Extrakten, kann als Kontrolproben angesehen werden. Wenn man alle positiven und alle negativen Serien in zwei Gruppen sammelt, erhält man die Zahlen 456:137 (30 %) und 437:0. Das beweist Statistisch, dass die positiven Resultater nicht auf reinem Zufall beruhen können.

Die Versuche haben gezeigt, dass die Resultate, die man mit heterozoischem Material erhält, verglichen werden können mit denen, welche man mit homozoischem erhält.

Es würde verfrüht sein, zu versuchen ein chemische Klassifikation des osteogenetischen Faktors auf den hier erhaltenen Versuchen zu begründen. Gewisse Schlussfolgerungen kann man jedoch ziehen. Es ist interessant, dass man nicht den gesammten aktiven Faktor eines Alkoholextraktes mit Hilfe von Benzen extrahieren kann, und dass die spezielle Solvens für Sterol und Lipoid, Petroläther, nicht imstande ist den aktiven Faktor vom Alkohol zu extrahieren. Dies unterstützt die Ansicht dass der aktive Faktor nicht ein reines Sterol oder Lipoid ist, welche Ansicht auch durch die geringe Aktivität der reinen Petrolätherextrakte unterstützt wird. Ausserdem gaben die aktivsten Extrakte negative Reaktionen, wenn untersucht auf Sterole. Die Aktivität der Azetonpräzipitate von Alkoholextrakten deutet auf Phosphatide hin, aber dagegen spricht die Löslichkeit der Präzipitate in destiliertem Wasser und ihre relative Unlöslichkeit in Öl.

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